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The Plant Mediator Complex in the Transcriptional Response to Low Temperature.

Charlotte Hannah Hurst

Supervisor: Dr. Heather Knight

Degree: Master of Science



**School of Biological and Biomedical
Sciences,
Durham University
2014**

“And now I think I am quite ready to go on another adventure. Are you coming?”

**Bilbo Baggins,
The Return of the King**

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Abstract

Title: The Plant Mediator Complex in the Transcriptional Response to Low Temperature

Author: Charlotte Hannah Hurst

SENSITIVE TO FREEZING6 (SFR6) is the MEDIATOR16 (MED16) subunit of the plant Mediator complex and is shown here to be required for the recruitment of Pol II and the Mediator complex to cold-inducible C-repeat binding factor (CBF)-controlled genes. In addition, the MED2 and MED14 subunits are also required for Pol II recruitment to these genes. Mutant lines impaired in expression of SFR6/MED16, MED2 and MED14 subunits showed impaired expression of many, but not all cold-inducible genes. Some cold-inducible genes that do not contain C-repeat element (CRT) motifs in their promoters were also misregulated in *sfr6/med16*, *mediator2 (med2)* and *mediator14 (med14)* mutant lines but Pol II recruitment was not impaired, unlike the situation for CRT-containing genes. Expression of cold-inducible genes was not impaired in all of the Mediator tail subunit mutant lines tested; NRB4/MED15 mutants were not impaired in their expression of cold-inducible genes but preliminary result suggested that this subunit might be involved in UV-induced gene expression. In addition to their role in the transcriptional response to cold, both SFR6/MED16 and MED14 subunits were shown to be required for the expression of known CBF-controlled cold-inducible genes in response to sugar, but the MED2 subunit was not. Preliminary experiments conducted on CDK-8 domain subunit mutant lines, CYCC and CDK8, indicated that the CDK-8 domain may not function solely as a transcriptional repressor but may be required for expression of dark- and UV-inducible genes. Together, these data illustrate that transcriptional control in plants is achieved through the combined action of subsets of Mediator subunits that are defined by the stimulus and the particular gene investigated.

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I. Introduction

Plants are constantly exposed to changing environmental conditions. When these changes are rapid or extreme, plants perceive them as a stress. As plants are unable to move to more favourable conditions, they have at their disposal sophisticated sensing mechanisms and signal transduction systems that allow the plant to respond and adapt to the stress so that it can continue to survive in its current environment. As shown in Figure I.1 below, the ultimate outcome of any stress such as cold, heat, nutrient deficiency or osmotic stress is an alteration in transcriptional regulation where different sets of genes are induced or repressed in response to the stress (Figure I.1). Altered gene expression leads to biochemical, physiological and morphological adaptations that will allow the plant to acclimate to the stress and survive (Krasensky & Jonak 2012).

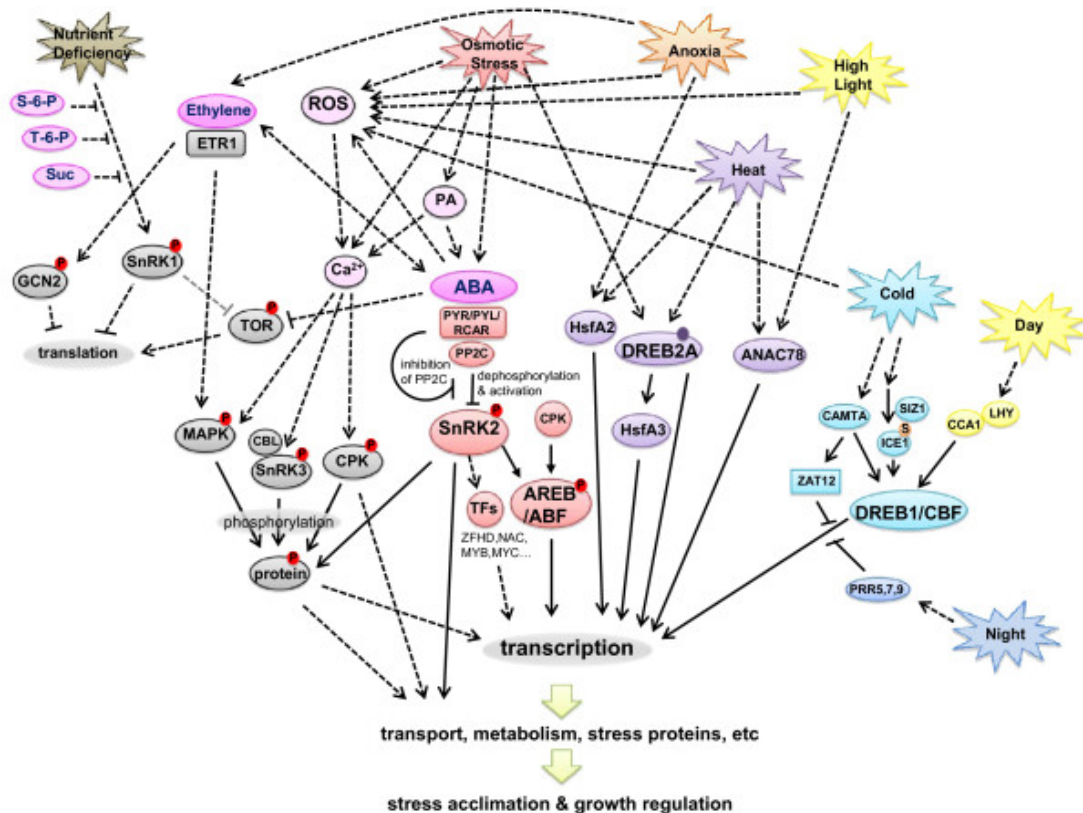


Figure I.1: Plant acclimation to abiotic stress requires a new state of cellular homeostasis that is achieved through signalling pathways that ultimately leads to changes in transcription. Control of gene expression in the abiotic stress response is tightly regulated by many cellular factors to maximize plant survival in response to a stress. (Cramer et al., 2011)

Stress responses act over different timescales: rapid post-translational effects such as phosphorylation, ubiquitylation or S-acylation (Seo & Lee 2004) provide immediate responses, activating stress-response signalling pathways that will lead to altered regulation of gene expression. Changes in gene expression occur more slowly than post-translational effects and result in metabolic and developmental alterations to the plant which are essential for a plant's long-term adaptation to the stress. These metabolic and transcriptional alterations are all reversible, affording the plant a large degree of plasticity in its ability to return to a basal state of homeostasis once the stress is removed.

I.1. The process of cold acclimation

Cold temperatures are one of many abiotic stresses faced by plants through the course of their lifecycle (Figure I.1). The ability of a plant to tolerate low temperatures is a major determinant of its global distribution and use as a crop. Understanding how plants respond to low temperatures is therefore important for protecting crop yields, increasing useable land area, extending growing seasons and to ultimately breed varieties of crops that can adapt more rapidly to environmental stresses and tolerate more extreme conditions.

Most temperate plants can survive mild chilling (temperatures $>10^{\circ}\text{C}$) and can be described as either freezing sensitive or freezing tolerant. Plants that are freezing-sensitive are commonly found in the tropics and sub-tropics and undergo chilling stress when temperatures fall below 10°C (Levitt 1980). Excessive chilling causes freezing-sensitive plants to wilt, undergo chlorosis and may even result in their death unless they have been acclimated by a gradual exposure to lower temperatures. Freezing-sensitive plants are unable to survive sub-zero temperatures even when they have been acclimated. Freezing tolerant plants are more commonly found in temperate regions; for them to survive through the winter months they must alter their transcriptional regulation and adjust their metabolism and physiology to deal with the sub-zero temperatures. This process is called cold acclimation and results in a significant increase in a plants tolerance to freezing temperatures (Thomashow, 1999). This is achieved by transcriptional and biochemical changes in response to a prior prolonged exposure of days or weeks to low, non-freezing temperatures between $0-5^{\circ}\text{C}$ (Warren et al., 1996).

In response to cold temperatures, a well-characterised series of physical and transcriptional changes occur within the plant cell that allows it to cold acclimate (Figure I.2): Cold temperatures rigidify the plant cell membrane (Orvar et al., 2000), resulting in an increase in cytosolic calcium concentrations ($[Ca^{2+}]_{cyto}$) and a reorganisation of the cytoskeletal networks (Knight et al., 1996, Orvar et al., 2000, Mazars et al., 1997). A series of mitogen-activated protein kinase (MAPK) proteins are also activated in phosphorylation cascades by cold that are thought to be involved in changes to gene expression independently of Ca^{2+} signalling. Overexpression of the MKK2 protein, which is normally activated in cold-induced MAPK cascades, under ambient conditions leads to the induction of many cold-inducible genes (Teige et al., 2004). C-Repeat Binding Factor (CBF) transcription factors are activated in response to cold, binding to the C-repeat (CRT) CCGAC promoter motif found in some but not all cold-inducible genes to activate them, allowing the plant to cold acclimate and become freezing tolerant (Figure I.2).

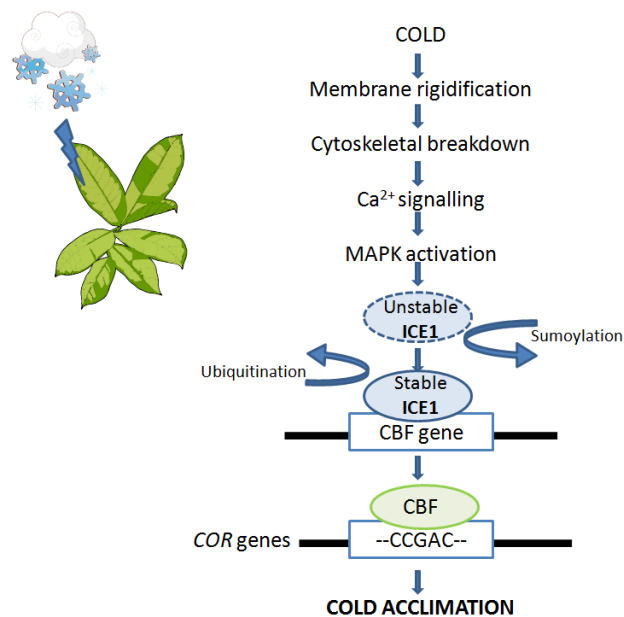


Figure I.2: Model for signalling leading from cold to the expression of *COR* genes and cold acclimation, regulated by CBF transcription factors in Arabidopsis. Ca^{2+} : calcium ion, MAPK: mitogen-activated protein kinase, ICE: Inducer of CBF Expression, CBF: C-repeat Binding Factor, *COR* genes: Cold-On Regulated genes, CCGAC: C-Repeat promoter element sequence. (Based on Knight & Knight 2012)

CBFs are the most well-studied pathway leading to altered transcriptional regulation of cold-inducible genes. However, some genes that do not contain CBF binding sites are also induced in response to cold, suggesting that non-CBF-regulated mechanisms of

cold-induced gene activation may exist (Hemsley et al., 2014). The function of many of these cold-responsive genes is still unclear, but some have been shown to be directly involved in protecting the plant from the stress, encoding genes of osmoprotectants, transporter proteins, detoxifying enzymes (Fowler & Thomashow 2002), regulatory proteins and transcription factors (Hannah et al., 2009).

The production of compatible solutes and osmolytes reduces the impacts of osmotic stress as water is drawn out of the cell during freezing and increases the osmotic potential of the cell, reducing damage from ice crystal formation within the cytosol (Steponkus 1984). However, when water in intercellular compartments freezes, it causes a reduction in water potential that leads to water movement from the cytoplasm where it is not frozen out to the intercellular space. This causes a drought-like response to the plant cell as less water becomes available for cellular processes (Steponkus et al., 1984). Osmolytes in the form of soluble carbohydrates accumulate during cold acclimation (Levitt, 1980, Pollock, 1984) to reduce cellular dehydration during freezing and act as a source of nutrition as photosynthesis is downregulated during cold acclimation (Hannah et al., 2005). It has been suggested that sugars may regulate cold acclimation (Guy et al., 1980) Research shows that treating dark-grown plants or plant cell cultures with exogenous sucrose can induce freezing tolerance (Tumanov & Trunova 1957, Tabaei Aghdaei et al., 2003). However, this effect was not seen in whole, light-grown seedlings, indicating that light strongly affects cold acclimation processes and sugar signalling networks, potentially by orchestrating sugar translocation around the plant (Steponkus & Lanphear, 1967). This suggests that feed-forward and feedback mechanisms exist in cold acclimation signalling pathways, allowing metabolic pathway products to regulate their own synthesis and act as regulators of other signalling pathways (Rekarte-Cowie et al., 2008). Thus sugars and other osmolytes play a dual role in the process of cold acclimation, indirectly through their use in plant metabolism and more directly in cryoprotection as regulators of cold acclimation pathways (Uemura & Steponkus, 2003). In addition, genes encoding detoxifying enzymes such as the D1 protein (Aro et al., 1993, 2005) are important to protect cells from photo-oxidative stress and the photoinhibition that occurs when plants encounter abiotic stresses such as cold temperatures (Nishiyama et al., 2006; Takahashi & Murata, 2008). Cold temperatures inhibit D1-mediated repairs to photosystem II (PSII) caused by ROS after light-induced damage (for review, see Nishiyama & Murata, 2014).

The plasma membrane is the primary site of signal transduction in a cell, where signals from the outside environment are transmitted into the cell for transcriptional responses to occur. As no cold receptor has yet been identified in plants, changes in membrane fluidity in response to low temperature led to the theory that membranes themselves act as 'thermometers', alerting the cell to temperature changes and activating cold-induced gene expression (Murata & Los, 1997, Los & Murata, 2000). Membranes are highly dynamic in response to cold stress as they are a major site of possible damage in plant cells during freezing temperatures (Webb et al., 1994). As water is lost from non-acclimated cells during freezing, this causes the cell to shrink and the excess membrane to be removed by clathrin-mediated endocytosis into the cell, reducing surface area (Minami et al., 2009). Problems arise upon thawing when water rapidly moves back into the cell from the intercellular space, which can cause expansion-induced lysis of the plant cell if the cell membrane no longer has enough surface area to contain the volume of water (Uemura & Steponkus, 1989). Cell membranes in plants that are not cold acclimated are also susceptible to damage via fracture jump lesions where the plasma membrane fractures and becomes continuous with various endomembranes resulting in "leaky" membranes that are unable to retain osmotic potential, resulting in cell death when it defrosts (Webb & Steponkus, 1993). Non-bilayer lipid structures such as lamellar to hexagonal II phase transitions of membrane phospholipids can also occur when the plasma membrane is brought in close proximity to chloroplast membranes as a result of freezing-induced cellular dehydration, which negatively impacts the permeability of the membrane and the viability of the cell (Gordon-Kamm & Steponkus, 1984).

To combat the possibility of plasma membrane damage, the composition of the plasma membrane changes significantly during the process of cold acclimation (Thomashow et al., 1999). An increase in membrane vesicle trafficking during cold acclimation results in an increased in fatty acid chain desaturation and phospholipid content of the plasma membrane, both of which have been correlated with an increase in membrane stability and freezing tolerance in plants (Steponkus, 1984, Uemura et al., 1995). Detoxifying enzymes are also important to remove reactive oxygen species (ROS) produced as a result of membrane damage caused by freezing. Membrane damage leads to the uncoupling of photosystems and the uncontrolled release of ROS into the cytosol as the electron transport chain, a major site ROS generation within the cell, becomes disrupted. In small amounts, ROS acts as part of stress signalling networks (Figure I.1)

but in larger quantities, can causes an oxidative stress on cellular components that can result in cell death (Gadjev et al.,2006). It has been shown that genes encoding photosynthetic components are down-regulated in response to cold temperatures, possibly to protect the cell from the greater risk of ROS-induced photo-oxidative damage for the duration of the stress (Stitt & Hurry, 2002, Hannah et al., 2005).

I.1.1. C-Repeat Binding Factor (CBF) transcription factors

Just as plants do not exist in isolation but in a complex environmental system, so DNA does not exist as a naked double helix of nucleic acids in the nucleus, but is decorated with a multitude of proteins and protein complexes. These proteins act in concert in a dynamic manner to regulate transcription by altering DNA packing (Strahl & Allis, 2000) and form the transcriptional machinery that allows specific gene activation in response to environmental signals. This includes histones, transcription factors such as CBFs which bind specific promoter elements, the enzyme RNA polymerase II (Pol II) which produces mRNA transcripts from the DNA sequence, and the Mediator complex which links these transcription machinery components together to activate genes (Conaway & Conaway 2011).

C-repeat binding factors (CBFs) are transcription factors that bind specifically to a CCGAC sequence in the promoter region of cold-responsive genes to activate them (Stockinger et al., 1997, Liu et al., 1998). Arabidopsis has a family of 3 genes encoding CBF transcription factors that lie in tandem on chromosome 4 (Liu et al., 1998, Gilmour et al., 1998). Transcripts of *CBF1*, 2 and 3 are rapidly upregulated by low temperature and show overlapping effects on *Cold On-Regulated (COR)* gene regulation (Gilmour et al., 2004). However, as not all cold-inducible genes are regulated by CBFs, the term 'COR genes' refers here only to those cold-inducible genes regulated by CBF transcription factors. *CBF* transcripts are relatively unstable at ambient temperatures with a half-life of 10 minutes, but can stably accumulate under cold conditions (Zarka et al., 2003). Overexpression of CBF transcription factors confers constitutive freezing, drought and salinity tolerance in Arabidopsis (Jaglo-Ottosen et al., 1998, Liu et al., 1998, Gong et al., 2002) but negatively affects both growth and development of the plant causing slowed growth, delayed flowering and reduced seed production (Liu et al., 1998, Kasuga et al., 1999, Gilmour et al., 2000). A *CBF4* gene also exists but is involved in drought and ABA responses rather than cold (Haake et al., 2002). In Arabidopsis, *CBF1-3* transcripts are

negatively regulated by MYB15, a transcription factor that binds to MYB-recognition elements in the *CBF1-3* gene promoters, repressing their transcription under ambient conditions (Agarwal et al., 2006). CBF2 has also been shown to negatively regulate expression of *CBF1* and *CBF3*, where *cbf2* mutants showed an elevated and more sustained expression of *CBF1*, *CBF3* and downstream *COR* gene transcripts (Novillo et al., 2004, 2007).

Transcription of the three *CBF* genes is positively regulated by ICE1 and ICE2 transcription factors. Under ambient conditions, ICE1 is ubiquitinated by HOS1 and degraded to prevent inappropriate CBF-mediated *COR* gene expression (Dong et al., 2006). Under cold conditions, ICE1 becomes sumoylated by SIZ1 to prevent ubiquitin-mediated degradation and activates transcription of *CBF1*, 2 and 3 to allow cold acclimation processes to occur within the plant (Miura et al., 2007) (Figure I. 2). DROUGHT-RESPONSE ELEMENT2 (DREB2A & DREB2B) transcription factors which are expressed in response to drought stress can also bind to the CCGAC promoter motif to activate expression of *COR* genes, thus it is described as the C-REPEAT/DROUGHT RESPONSE ELEMENT (CRT/DRE) promoter motif (Yamaguchi-Shinozaki & Shinozaki, 1994; Thomashow, 1999). It is likely this occurs because freezing and drought stress are both osmotic stresses: Ice formation in intercellular spaces draws water out of the cytosol, reducing the volume of water available for cellular resulting in osmotic stress (Steponkus 1984). During drought stress, water is lost from cells by transpiration and is unable to be replaced, causing an osmotic stress on the plant. Thus the *CBF1*, *CBF2* and *CBF3* genes are also known as *DROUGHT-RESPONSE ELEMENT1B*, *C* and *A* (*DREB1B*, *DREB1C* and *DREB1A*) respectively as they are responsive to both cold- and drought-stress (Liu et al., 1998).

Research has shown that *CBF1-3* transcripts accumulate not only in response to cold temperatures, but also in response to touch, abscisic acid (ABA) and the circadian clock (Gilmour et al., 1998; Knight et al., 2004; Fowler et al., 2005), suggesting that they may play a role in gene regulation in response to other stresses than cold. Fowler showed that *CBF3* transcript levels are gated by the circadian clock, normally cycle throughout the day (Fowler et al., 2005) with *CBF3* transcript levels accumulating to maximum levels early in the morning and reach minimum levels in the early evening period in non-cold-treated plants (Harmer et al., 2000). The accumulation of *CBF1*, *CBF2* and *CBF3* transcripts is also gated in cold-treated plants, with their expression being dampened

during the evening period and at the lowest levels of expression during the night (Fowler et al., 2005). *CBF* transcripts are also known to be regulated by circadian clock gene transcription factors, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED1 (CCA1) as *lhy* and *cca1* mutants are unable to correctly express *CBF*-regulated genes (Dong et al., 2011). As plants can detect a change in temperature of just 1°C (Knight et al., 2000), this suggests that the circadian clock is also an important factor in cold-induced gene regulation, enabling the plant to distinguish between the cooler temperatures at night which do not require a full cold-acclimation response and low daily temperatures of <10°C that signal the approach of winter for which cold acclimation is a vital response (Bieniawska et al., 2008). However, much research is needed before we can gain a fuller understanding of how plants distinguish between these environmental cues to effect the appropriate response.

I.2. The Mediator Complex

While much is known of the role of transcription factors and Pol II in transcription, the Mediator complex is only in recent years being recognised as an essential component in many aspects of plant responses to their environment. The Mediator complex was originally purified from yeast (*Saccharomyces cerevisiae*) and mammalian cells (Guglielmi et al., 2004, Kang et al., 2001) however the plant Mediator complex has only recently been isolated, thirteen years after its initial discovery, so studies investigating the precise roles of the different subunits in plants are still being undertaken.

Named for the intermediary role it plays linking Pol II to transcription factors, Mediator provides an additional level of discrimination amongst environmental signals to activate genes allowing eukaryotes to correctly and effectively respond to specific signals (Conaway & Conaway 2011). Mediator has no counterpart in bacteria and represents a new layer of complexity between Pol II and regulatory proteins unique to eukaryotes. This additional layer of complexity could account for the greater capacity for cell differentiation and development of eukaryotes as a result of the increased complexity of gene regulation (Kornberg, 2005).

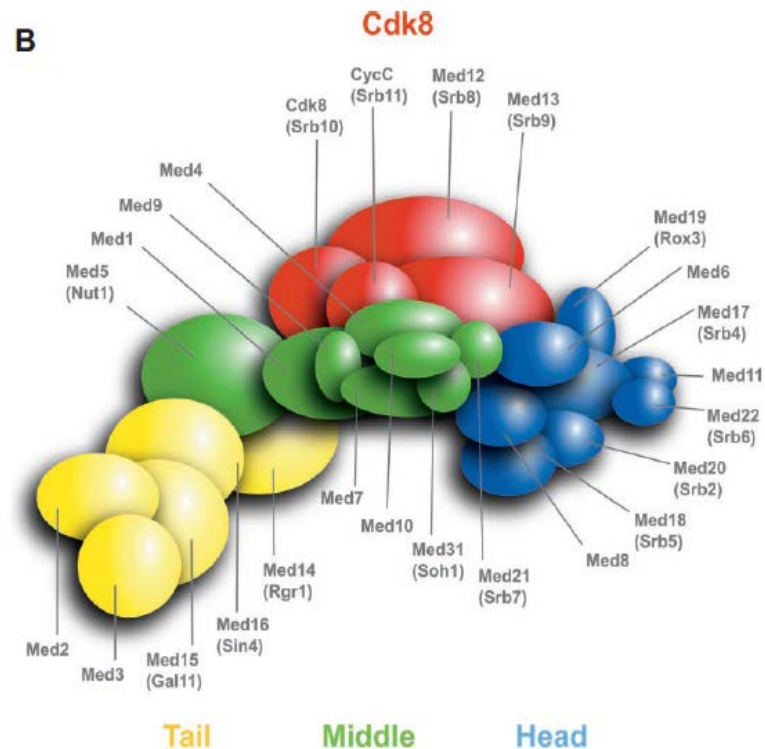


Figure I.3: Cartoon diagram of proposed interactions of subunits in the yeast Mediator complex (Guglielmi *et al.*, 2004).

Mediator is a complex of 25-35 proteins (Figure I.3) that binds to both transcription factors and RNA Polymerase II (Kim *et al.*, 1994) (Figure I.4). Its structure in eukaryotes has been conserved during evolution, consisting of 4 domains: the head, the tail, the middle and the cyclin-dependent kinase 8 (CDK-8) domain. Even though the primary sequences of Mediator subunits have extensively diverged between species, the overall structure and function has been conserved (Guglielmi *et al.*, 2004). The head domain binds to RNA polymerase II and is highly conserved between species. The tail domain binds transcription factors and has diverged greatly between eukaryotes (Figure I.5). Mediator was originally purified from crude *S. cerevisiae* extracts and counterparts of yeast Mediator have been found in every eukaryote investigated (Boube *et al.*, 2002). The close correspondence between yeast and mammalian Mediator was initially shown by structural studies and conservation was established by genomic and proteomic analysis (Sato *et al.*, 2004, Bourbon *et al.*, 2008). With the isolation of the Mediator complex, the proteins identified in previous screens for mutations affecting transcriptional regulation in yeast (Gustafsson *et al.*, 1997) were united in a common biochemical entity (Kornberg, 2005).

Early research on Mediator led to the theory that Pol II and Mediator were recruited together as a holoenzyme to gene promoters (Kim et al., 1994; Koleske & Young, 1994). However, research later showed that Mediator and Pol II are recruited independently and that Mediator recruitment occurs prior to Pol II recruitment (Bryant & Ptashne, 2003; Kuras et al., 2003). *In vitro* experiments have also revealed that Mediator may play a role both in histone occupancy, Pol II reinitiation (Yudkovsky et al., 2000) and after transcriptional initiation (Wang et al., 2005), suggesting that Mediator has various modes of transcriptional activity depending on the gene investigated. The plant Mediator complex has only relatively recently been purified, compared to the yeast counterpart. It was purified from *Arabidopsis* suspension culture (Bäckström *et al.*, 2007) and contained most of the components present in the core complex in other organisms, but was missing the CDK8 domain. This led to two hypotheses about Mediator, where forms of Mediator with different assortments of subunits were recruited depending on the gene being activated or that when Mediator was isolated with Pol II, it did not have the CDK-8 domain attached, leading to the conclusion that the kinase domain was only present under repressive conditions (Bäckström *et al.*, 2007).

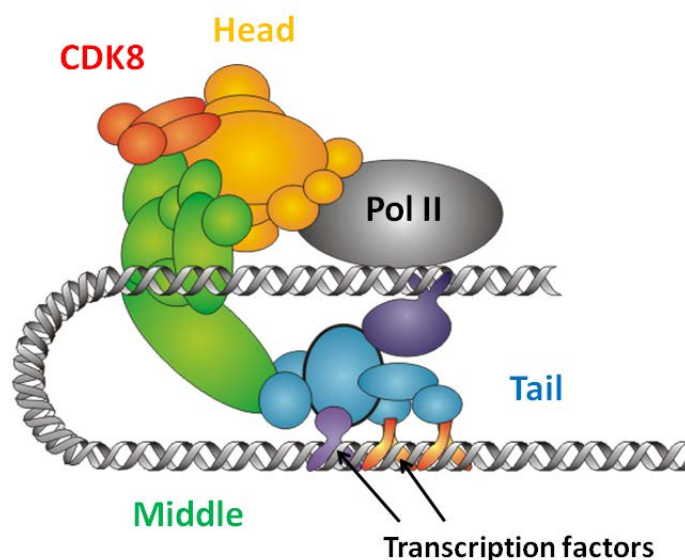


Figure I.4: Diagram of the proposed plant Mediator complex structure linking transcription factors such as CBFs bound to promoter elements to the RNA Pol II enzyme to activate gene transcription. Pol II: RNA polymerase II. (Image from P. Hemsley, Personal Comm.)

I.2.1. The plant Mediator complex

At the amino acid sequence level, homology of the plant Mediator subunits to other eukaryotes is relatively low, at around 30% homology at best (Bäckström *et al.*, 2007). Because of this, it was originally thought that plants had several unique subunits, however *in silico* studies have since shown that some of these subunits have homologues in yeast Mediator but have diverged so greatly in sequence that they were not, at first, recognisable (Bourbon *et al.*, 2008). Using a different bioinformatic approach to Bäckström (2007), Bourbon *et al.* (2008) showed that subunits such as MED32 and MED33 were homologous to previously identified yeast Mediator subunits. Whilst the original study by Bäckström identified 6 Mediator subunits that were unique to plant Mediator, this was in fact an overestimation (Bäckström *et al.*, 2007). It has since been shown that of those original 6, MED32 is homologous to the yeast MED2 tail domain subunit and MED33 is equivalent to yeast MED5 head domain subunit, leaving just four subunits of the plant Mediator complex that are thought to be unique (Bourbon *et al.*, 2008).

I.2.2. Specific functions of plant Mediator subunits

Sixteen of the proposed 34 plant Mediator subunits have either been assigned a function or have a defined phenotype for the gene-specific mutations (Kidd *et al.*, 2009). Plant Mediator subunits have been shown to regulate a variety of functions in plants, including the response to abiotic and biotic stresses, flowering times and developmental control, DNA helicase activity, genome stability, RNA processing and non-coding RNA production. The remaining 11 subunits are yet to be characterised.

I.2.2.1. Head domain

The head domain of Mediator is the most evolutionarily conserved of the 4 domains as it serves as the binding site of Pol II to activate gene transcription. The MED8 (SETH10) subunit was characterised before the Mediator complex was identified. MED8 was found to regulate flower development as *med8* mutants exhibit delayed flowering times and a slower pollen tube growth rate under long day conditions due to reduced transcripts of key positive flowering gene regulators FLOWERING LOCUS T (FT) and CONSTANS (CO) (Lalanne *et al.*, 2004, Kidd *et al.*, 2009). MED8/SETH10 has now also

been shown to have an additive role with the PFT1/MED25 subunit in the plant defence responses against the leaf-infecting necrotrophic fungal pathogen *A. brassicola* (Kidd *et al.*, 2009). Mutations in the MED17, MED18 and MED20a subunits all have similar phenotypes and have been shown to regulate plant development, non-coding RNA production and display reduced miRNA levels (Kim *et al.*, 2011). The MED18 subunit has also recently been shown to play a positive role in the resistance to fungal infection by the necrotroph *B.cinerea* interaction by interacting with the YIN YANG1 (YY1) transcription factor to induce *TRX* and *GRX* genes that provide resistance to *B. cinerea*. However, known markers of immune response pathways such as *R*-genes and jasmonic acid-response genes show no altered expression in *med18* mutants suggesting a new plant immunity pathway that functions distinctly from other previously-identified plant immune responses (Lai *et al.*, 2014). In addition to its involvement in biotic stress responses, Lai *et al.* (2014) also showed that the MED18 subunit is involved in abiotic stress responses, phytohormone signalling and flowering. MED18 interacts with the ABI4 transcription factor to positively regulate genes induced in the response to ABA, and interacts with the SUF4 transcription factor for normal expression of the floral repressor gene, *FLOWERING LOCUS C (FLC)*. Mutations in SUF4 result in early flowering due to reduced expression of *FLC*, suggesting the MED18 normally suppresses SUF4 function to enable expression of *FLC* (Lai *et al.*, 2014). A role for MED35 and MED36 subunits has not yet been shown in plants, however Kang showed that the yeast MED35 subunit is involved in pre-mRNA processing and could bind to the C-terminal domain (CTD) repeats of the largest RNA pol II subunit (Kang *et al.*, 2009). In mammalian systems, the MED36 (FIB2) subunit encodes a fibrillarin which is a key nuclear protein in eukaryotes that is responsible for regulating the methylation and cleavage of rRNA (Huang *et al.*, 2009).

1.2.2.2. Middle Domain

The MED5b/MED33b/REDUCED EPIDERMAL FLUORESCENCE4 (REF4) subunit is required for uncompromised accumulation of phenylpropanoid pathway products which provide plants with UV, pathogen and herbivore defences. Research has shown *ref4* mutants are also rendered partially dwarfed due to impaired lignin biosynthesis (Stout *et al.*, 2008). Interestingly, the REF4 (MED5b) subunit is also predicted to be a transmembrane protein, adding a new dimension to the cellular localisation and functions of the plant Mediator complex (Stour *et al.*, (2008). Recent work from our lab has shown that

MED5a and MED5b are functionally redundant, and that the MED5/REF4 subunit is required for dark-induced gene expression in double *med5a/med5b* mutants (Hemsley et al., 2014). The MED21 subunit has been implicated in both biotic stress responses and development in plants. Interestingly, homozygous *med21* subunit mutants show an embryo lethal phenotype in both plants and mice (Tudor et al., 1999, Dhawan et al., 2009), thus research on *med21* mutants in plants had to be carried out on heterozygous MED21/*med21* plants. Dhawan also showed that the MED21 subunit regulates plant defences in response to the necrotrophic pathogens *A. brassicola* and *B. cinerea* via an interaction with the HUB1 (HISTONE MONOUBIQUITINATION1) protein to induce jasmonate acid-responsive defence genes (Dhawan et al., 2009).

It is unclear whether the MED25 subunit is located in the middle domain of the plant Mediator complex as yeast, the organism on which the interaction map is based (Figure 1.3), does not contain a MED25 subunit. However, from research carried out on the mammalian Mediator complex, the plant MED25 subunit is predicted to form part of the middle domain (Tomomori-Sato et al., 2004). The MED25 subunit was first identified as a positive regulator of shade avoidance called PHYTOCHROME AND FLOWERING TIME 1 (PFT1) (Cerdán & Chory, 2003) that acts downstream of phytochrome B in the phytochrome signalling pathway to promote flowering under shaded conditions. However it is now considered to be a negative regulator of the phytochrome signalling pathway rather than a component of it as *pft1* mutants are not impaired in flowering under shaded conditions (Wollenberg et al., 2008). MED25/PFT1 also has a role in the biotic stress response, acting as a positive regulator of jasmonic acid signalling during infection by fungal pathogens such as *A. brassicola* and *B. cinerea* as *med25/pft1* mutants show increased susceptibility to these fungi but shows resistance to *F. oxysporum*, a root-infecting hemibiotrophic fungal pathogen (Thatcher et al., 2009). The previously described *med8* mutants also showed an altered flowering time and susceptibility to *A. brassicola* as *med25/pft1* mutants do, however double *med8pft1* mutants showed even later flowering times than single mutants and increased resistance to *F. oxysporum*, suggesting that the MED25/PFT1 and MED8 subunits have additive functions in biotic stress responses and flowering. The MED25 subunit has been shown to interact with the MYC2 transcription factor to negatively regulate the ABA response during seed germination and (Chen et al., 2012). Recently, the MED25/PFT1 subunit has also been shown to interact with 8 different transcription factors (Ou et al., 2011), three of which induce expression of *PLANT DEFENSIN1.2* (*PDF1.2*), a marker gene

of activated jasmonic acid signalling. It has been suggested that *med25/pft1* mutants may not be able to correctly interact with these transcription factors, causing the reduced jasmonic acid-associated gene induction (Figure I.5). MED25/PFT1 has also been implicated in many aspects of plant abiotic stress responses and development (Elfving *et al.*, 2011). Research has shown that the MED25/PFT1 subunit interacts with ZFHD1 and DREB2A, transcription factors that are involved in abiotic stress tolerance (Tran *et al.*, 2006, Sakuma *et al.*, 2006) (Figure I.5). Interestingly, *med25/pft1* mutants are more drought-resistant than wild-type plants but, are more salt sensitive, which is unusual as these stress responses are highly interlinked (Elfving *et al.*, 2011). Elfving (2011) showed that *med25/pft1* mutants, and mutated salt-responsive transcription factors *zfhd1* and *myb-like* that interact with MED25 to confer salt tolerance. However, while *dreb2a* mutants are more susceptible to drought, *med25/pft1* mutants are more drought-resistant due to massive upregulation of *DREB2A* transcripts. This suggests that MED25 functions downstream of MYB-like and ZFHD1 transcription factors to activate expression of genes encoding proteins required for salt stress responses (Figure I.5). However, DREB2A interacts with MED25, acting as a transcriptional repressor in response to drought stress as *dreb2a* mutants are less salt-sensitive than *med25/pft1* mutants (Elfving *et al.*, 2011).

The MED34 (RECQ HELICASE2) subunit is a DNA helicase that is important for genome stability. Mutations in the MED34 subunit can disrupt D loop structures in DNA, impeding DNA repair mechanisms and telomere stability. MED34 also mediates branch migration of Holliday junctions, impacting homologous recombination during meiosis (Kobbe *et al.*, 2009, 2010). The final subunit that has been characterised in the middle domain of the plant Mediator complex is MED37a/BIP1 (Jin *et al.*, 2007), which is a member of the HSP70 chaperone family and is homologous to the yeast Ig binding protein interacts with the brassinosteroid hormone receptor, BRI1 (Hong *et al.*, 2008). BRI1 is a leucine-rich repeat (LRR) receptor-like kinase (RLK) protein that is embedded in the plasma membrane and acts as a receptor for brassinosteroid phytohormones which are crucial for plant growth and development (Kinoshita *et al.*, 2005, Li & Chory 1997, Clouse & Sasse 1998). During BRI1 signalling, MED37a/BIP1 interacts with BRI1 to be degraded by a proteasome-independent endoplasmic reticulum-associated cellular mechanism (Hong *et al.*, 2008)

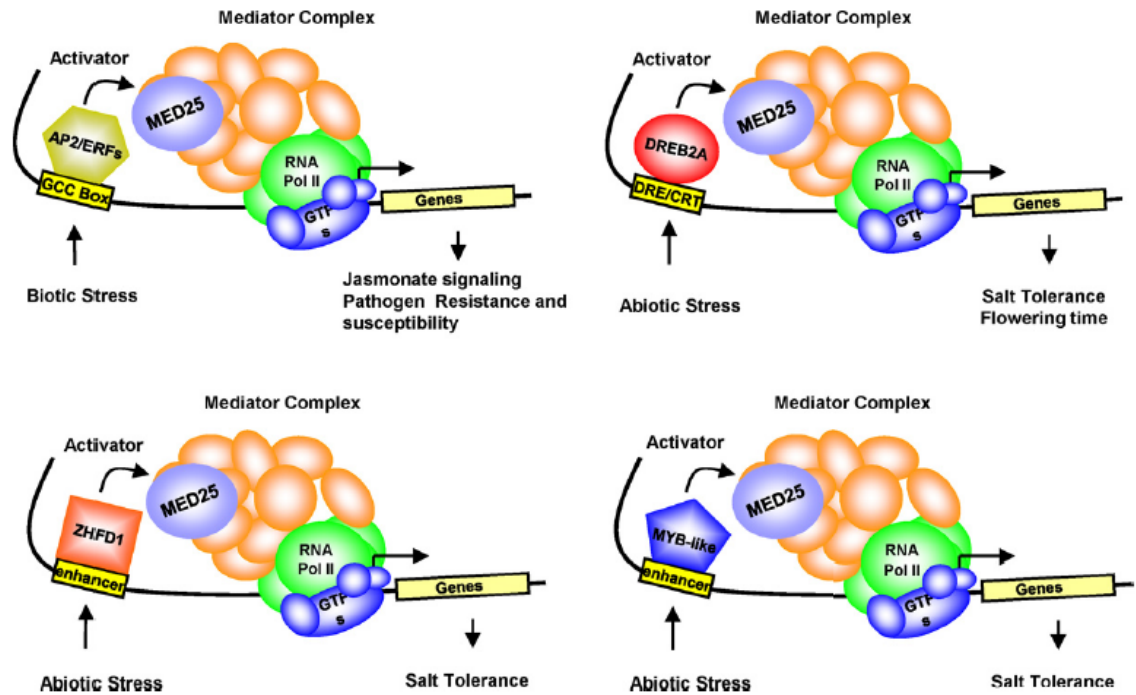


Figure I.5: Arabidopsis MED25 integrates multiple abiotic, biotic and developmental pathways through interactions with different activators. From Kidd et al., 2011

I.2.2.3. CDK-8 domain

The CDK-8 domain is perhaps the most poorly understood of the 4 Mediator domains and much conflicting data from mammalian, yeast and plant Mediator experiments exists on the function of CDK-8 domain subunits. In plants, the CDK8 domain is composed of 4 subunits, CYCC, CDK8, MED12 and MED13 that have homologues in mammalian and yeast Mediator (Samuelson *et al.*, 2003, Loncle *et al.*, 2007). Research carried out on mammalian Mediator led to the conclusion that the CDK-8 domain has a repressive function on Mediator activation when it is bound to the complex (van de Peppel *et al.*, 2005). Research showed that the CDK-8 domain prevents Mediator associating with RNA polymerase II and promotes epigenetic silencing of target genes via chromatin methylation (Ding *et al.*, 2008). Conflicting research shows both transcriptional activation (Holstege *et al.*, 1998) and repression (Carlson, 1997) of sugar-responsive genes in mammalian cells with mutations in SRB10, the mammalian homologue of the CKD8 subunit. In yeast and animals, MED12 and MED13 subunits have also been shown to act as transcriptional repressors, inhibiting the core Mediator function by complexing with homologues of CDK8 (SRB10) and CYCC subunits (Andrau *et al.*, 2006). The developmental phenotypes of *med12* and *med13* mutants in *Drosophila* and *C. elegans* are consistent with expression of homologous genes in yeast in

microarray analysis, indicating that they positively regulate only a small number of developmental genes (Samuelsen *et al.*, 2003), compared to the rest of core Mediator which regulates nearly all transcription events (Kornberg, 2005).

However, research is now emerging to challenge the original hypothesis that the CDK-8 domain acts as a general repressor of transcription. In human tumor cell lines the CDK-8 domain is shown to be a positive regulator of genes by its interaction with transcription elongation factors (Donner *et al.*, 2010). In addition, mammalian CDK-8 domain subunits KIN28 and CDK8/SRB10 have been shown to promote Pol II-mediated transcription and formation of the scaffold complex (Liu *et al.*, 2004). In plants, the MED12 (CENTRE CITY CCT) and MED13 (GRAND CENTRAL/MACCHI-BOU2) subunits are required for correct embryo development and patterning (Gillmor *et al.*, 2010; Ito *et al.*, 2011) as was found in *Drosophila* and *C. elegans* (Samuelsen *et al.*, 2003). This suggests that the CDK-8 domain could play a positive role in transcriptional regulation, rather than just a negative one. The CDK8/HEN3 subunit is required for correct floral organ development by interacting with a co-repressor called LEUNIG (Gonzalez *et al.*, 2007). Little is known about the CYCC subunit of the CDK-8 domain in plants

I.2.2.4. Tail domain

It is widely accepted that subunits of the tail domain contain sites for transcription factor binding, though no site-targeted mutagenesis of predicted binding sites on these subunits has yet been carried out in plants. It was originally hypothesised that the tail domain subunits were solely responsible for binding transcription factors, however research has now shown that subunits from other domains, such as MED18 and MED25/PFT1, are capable of binding different transcription factors to induce specific stress-response genes (Kidd *et al.*, 2011, Lai *et al.*, 2014), suggesting that the original proposed structure of the Mediator complex may be different to what occurs *in vivo* (Guglielmi *et al.*, 2004, Figure I.3 & Figure I.4).

Canet *et al.* (2012) found that only *nrb4/med15* point mutants were viable while T-DNA insertion lines were embryo lethal or sterile, suggesting that, like the MED21 subunit, the NRB4/MED15 subunit is essential to correct functionality of the plant Mediator complex and plant survival (Canet *et al.*, 2012, Dhawan *et al.*, 2009). Research showed that the NRB4/MED15 subunit is unique amongst plant Mediator subunits as being the first subunit that is responsible for mediating salicylic acid-dependent pathogenesis

responses in plants as MED25, MED8 and MED21 were all shown to be involved in jasmonic acid-mediated plant defence responses (Kidd et al., 2009, 2011, Dhawan et al., 2009). It is predicted that up to three NRB4/MED15 orthologues may exist in Arabidopsis with non-redundant functions (Mathur et al., 2011), as only one *nrb4* mutant show increased susceptibility to the plant pathogen *Pseudomonas syringae* and impaired salicylic acid responses, while the other two did not (Canet et al., 2012). The MED14 tail subunit was shown to be the previously identified STRUWWELPETER (SWP) protein (Clay & Nelson 2005) which dictates the duration of the cell proliferation phase in the leaf primordium but does not affect the rate of cell division, resulting in smaller cells and plant organs in *med14/swp* mutants (Autran et al., 2002). As a result, *med14/swp* mutants also show a disorganised shoot apical meristem (Clay & Nelson, 2005) and have also recently been shown to have altered pathogenesis responses to *Pseudomonas syringae* DC3000 with greatly reduced induction of many jasmonic acid-responsive pathogen-associated genes (Zhang et al., 2013). In yeast, the MED16/SIN4 and MED14/RGR1P proteins physically interact, deletion of the MED14/RGR1P subunit causes loss of the rest of the tail subunits MED2, MED3 and MED15, indicating that the MED14/RGR1P subunit of the yeast Mediator complex also plays a structural role, anchoring the other tail subunits on to the main Mediator complex (Li et al., 1995). The plant MED14 subunit may also play a similar role to the yeast RGR1P subunit based on its predicted location within the complex (Guglielmi et al., 2004, Figure1.3), but this has yet to be shown *in vivo*.

The MED16 subunit of the plant Mediator complex was first identified as the SENSITIVE TO FREEZING6 (SFR6) protein and was discovered in an EMS (ethyl-methanesulfonate) mutant screen of Arabidopsis plants that failed to cold acclimate and could not survive freezing temperatures (Warren et al., 1996). SFR6 was later shown to have impaired expression of many *Cold-On Regulated (COR)* genes such as *KIN2* and *GOLS3*, which contain the CRT/DRE promoter motif and are induced by CBF1-3/DREBA-C transcription factors in response to both drought and cold stress (Knight et al., 1999, Boyce et al., 2003). It was the impaired COR gene expression that led to freezing sensitivity of *sfr6* mutants, even after a period of cold acclimation (Knight et al., 1999). Expression of CBF transcription factors is not impaired in *sfr6/med16* mutants, showing that SFR6 acts downstream of CBF transcription factors to induce expression of *COR* genes in response to cold (Knight et al., 2009). The SFR6 protein was eventually identified as the MED16 subunit of the plant Mediator complex by Bäckström et al. (2007) and is homologous to

the SIN4 subunit of the yeast Mediator complex (Li et al., 1995). Much like the MED25/PFT1 subunit, SFR6/MED16 is responsible for biotic stress responses and regulation of flowering time pathways. *med16/sfr6* mutants were shown to be more susceptible to *Pseudomonas syringae* DC3000 as they were unable to correctly upregulate genes required for the salicylic acid-mediated defence response and the pathogenesis-related genes *PR1*, *PR2* and *PR5* in response to infection with *P. syringae*. In addition, *sfr6/med16* exhibit compromised jasmonic acid responses as mutant lines failed to express well-established jasmonic acid-response genes, *PDF1.1* and *PDF1.2A* genes in response to jasmonic acid treatment, indicating that both the MED25/PFT1 and SFR6/MED16 subunits are required for full expression of jasmonic acid-responsive genes (Wathugala et al., 2012).

The SFR6 subunit is also required for correct regulation of the circadian clock as *sfr6/med16* mutants show a delayed flowering phenotype and reduced sensitivity to day length compared to wild type and reduced expression of clock components *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *TIMING OF CAB1* (*TOC1*) and genes whose promoters contained circadian clock-associated promoter motifs. Given the role of sugar in the process of cold acclimation, it is unsurprising that *sfr6/med16* mutants also show a limited, but not completely absent response to treatment with sucrose (Knight et al., 2008). As a result of the delayed flowering phenotype, *sfr6/med16* mutants also have a lower seed set than wild type plants. In addition to the role that the SFR6/MED16 subunit plays in the biotic, abiotic stress response and flowering time pathway, induction of UV response genes is also impaired in *sfr6/med16* mutants (Knight et al., 2012). Mutant plant lines also show poor tolerance of UV-C irradiation and recovery after exposure. Many genes induced by UV-C are also salicylic acid-responsive and research has shown that many of these genes are misregulated in *sfr6/med16* mutants, suggesting that UV-C responses are mediated through salicylic acid-dependent gene expression and are regulated by the SFR6/MED16 subunit (Knight et al., 2012). Thus the SFR6/MED16 subunit of the plant Mediator complex, like MED26/PFT1 and MED21 subunits acts to integrate many stress response and developmental response networks in Arabidopsis to regulate gene expression and stress tolerance.

I.3. Aims of the thesis

In light of previous research highlighting the important role of MED16/SFR6 in the transcriptional response to cold, microarray experiments were carried out to further dissect expression of the genes regulated by MED16/SFR6 in response to cold. Work carried out in this thesis firstly validates this microarray data and explores whether the MED16/SFR6 subunit is required for expression of all CBF-regulated cold-inducible genes. Secondly, expression of cold-inducible genes that are not CBF-regulated were also investigated in *sfr6/med16* mutant lines to elucidate whether MED16/SFR6 is also involved in the activation of non-CBF-regulated genes. In addition, the role of other Mediator tail subunits MED2, MED14 and MED15/NRB4, predicted to be adjacent to MED16/SFR6 in the plant Mediator complex, are investigated in the transcriptional response to cold. This will be carried out using tDNA knock out and EMS plant lines subjected to cold or ambient conditions. This is to explore whether the MED16/SFR6 subunit plays a unique role in the expression of cold-inducible genes, or whether these other subunits are required, acting in concert, for correct cold-inducible gene expression.

Little is known about the function of the CDK-8 domain subunits CYCC and CDK8, conflicting data in the literature suggests a role for both transcriptional activation and repression of CDK-8 domain subunits. Therefore, the third aim of this thesis examines the transcriptional response to abiotic stresses such as cold, darkness and UV exposure in *med15/nrb4* and CDK-8 domain subunits *cycC-1*, *cycC-2* and *cdk8* Mediator mutant lines and elucidates whether specific subunits are required for the transcriptional response to abiotic stress, or whether individual subunits each regulate a different abiotic stress transcriptional response in Arabidopsis.

II. Materials and Methods

Recipes for all buffers and solutions used can be found in Appendix 1.

II.1 Plant Culture

II.1.1 Plant Lines

med14-1: insertion in At3g04740 (SAIL_373_CO7) (Zhang et al., 2013), *sfr6-1*: EMS point mutation leading to premature stop codon in At4g04920 (Knight et al., 1999). Insertion lines not previously published were obtained in the following genes from NASC (<http://signal.salk.edu/cgi-bin/tdnaexpress>): *MED2*: At1g11760 (SALK_028490), *CycC-1*: At5g48630 (SALK_039400), *CycC-2*: At5g48640 (SAIL_102_B02), *CKD8*: At5g63610 (SALK_072781C), *NRB4/MED15*: At1g15780.

II.1.2 Seed sterilisation

Seeds were sterilised in 70% ethanol (Fisher Scientific E/0650DF/25) with constant shaking for 10 minutes before being left to dry on 90mm filter paper (Whatman™ Filter Paper) in a laminar flow hood to prevent contamination.

II.1.3 Plant growth conditions

For all experiments described below, seeds were sown on autoclaved plant culture grade agar (SIGMA) with MS medium including vitamins (Murashige & Skoog, 1962) (Duchefa Biochimie M0222.0001), stratified at 5°C for 2 days after sowing to synchronise germination and transferred to either a Percival CU-36L5 or SANYO MLR-350 growth chamber with a 16h/8h light/dark cycle at 20°C (±1°C).

For DNA and RNA extractions, plants were grown for 8 days on 1x MS 0.8% agar in a Percival CU-36L5. For ChIP analysis, plants were grown for 3 weeks on 0.5x MS 1.5% agar in a SANYO MLR-350 growth chamber. For biolistic transformation, plants were grown in the centre (approx. 2cm diameter circle) of a 9cm plate containing 0.5x MS 0.8% agar for 8 days in a SANYO MLR-350 growth chamber. For protoplast extraction, plants were grown for 3 weeks on 1xMS 0.8% agar in a SANYO MLR-350 growth chamber.

II.1.4 Time course

Arabidopsis thaliana ecotype Columbia-0 seeds were grown as described previously for RNA extraction. Seedlings were transferred to constant light growth conditions at 20°C

24h prior to the experiment. Samples were taken at 3, 6, 9, 12 and 24h intervals after presumptive dawn from ambient- and cold-treated seedlings, to choose a suitable time point for *COR* gene expression. Tissue was frozen in liquid nitrogen and stored at -80°C until use.

II.2 Stress assays

II.2.1 Cold treatment

Seedlings were transferred to cold (5°C) or ambient (20°C) SANYO MLR-350 growth chambers with matching light conditions of 150 μ E/m²/s. Unless otherwise stated, tissue was harvested after 6h for RNA extraction or after 4h for ChIP analysis and flash frozen in liquid nitrogen and stored at -80°C until use. These timepoints were chosen based on data obtained from the previously described time course experiments.

II.2.2 Sugar/light/dark

Plants were grown as previously described for RNA extraction. After 8 days, seedlings were transferred to either 3% w/v sucrose or an isoosmolar equivalent concentration of mannitol (0.096M; control) and maintained in the light or covered in foil for darkness 3h after presumptive dawn. Tissue was harvested after 6h, briefly blotted dry on tissue to remove excess sucrose or mannitol solution and stored at -80°C for RNA extraction.

II.2.3 UV-C

Seedlings grown as previously described for RNA extraction were treated with 5KJ.m² UV-C (254 nm) at 8 days old in a UVITEC CL-E508.G cross-linker. The Perspex lid covering the seedlings was removed for UV treatment. After treatment, seedlings were returned to ambient conditions for a period of 24h before being harvested and tissue stored at -80°C until use.

II.2.4 Darkness

Seedlings were grown as described for RNA extraction and wrapped in two layers of tinfoil 3h after presumptive dawn at 20°C at 8 days old. Seedlings were then harvested in the dark and tissue frozen at -80°C until use.

II.3 Transformations

II.3.1 *E.coli* transformation

25µl of *E. coli* DH5α cells (Bioline Silver grade competency cells) were transformed using 1µl plasmid DNA. After addition of the DNA, cells were incubated on ice for 20 minutes before being heat shocked at 42°C for 45 seconds. The cells were briefly cooled on ice and 475µl of LB was added. The cells were incubated on a rocking platform at 37°C for 1h to allow recovery. The cells were plated out on LB agar plates containing the appropriate antibiotic and left at 37°C overnight. After 24h, a single colony was selected and used to inoculate 5ml LB containing antibiotic selection. The culture was then incubated at 37°C for 8h with vigorous shaking (300rpm). After 16h, 2ml of the starter culture was used to inoculate a 200ml LB culture. The plasmid was then extracted using the plasmid maxiprep kit as described below.

II.3.2 Biolistic transformation

II.3.2.1 *Preparation of the gold particles*

For the bombardment, 1.6µm gold microcarriers (Bio-Rad #165-2264) were used. 60mg of gold particles were washed three times with 1ml 100% ethanol and vortexed for 1min. The gold particles were then washed in 1ml nuclease-free water and finally resuspended in 1ml nuclease free water and stored at 4°C until use.

II.3.2.2 *DNA-coating the particles*

The pre-washed gold particles were fully resuspended by vortexing and 50µl was removed into a new Eppendorf. 5µg total of two high copy number plasmids was then added to the suspension and vortexed continuously for 30seconds. The plasmids used were a CRT concatamer reporter consisting of 4 copies of CRT fused to a luciferase (Whalley et al., 2011) and a 35S:: aequorin construct (Knight et al., 1991) for normalisation purposes. 50µl of 2.5M CaCl₂ was added and the particles were vortexed as before. 20µl of 0.1M spermidine-free base was then added and the gold particle suspension was placed in a continuous vortex for 3 minutes. The gold particles were briefly pelleted and the supernatant was removed. The DNA-coated gold particles were washed in 250µl of 100% ethanol and completely resuspended in 125µl 100% ethanol. Five large macrocarrier discs were inserted into their macrocarrier rings and 20µl of the fully resuspended DNA-coated gold particles was added to the centre of each macrocarrier disc. DNA-coated macrocarrier discs were left to dry under a Perspex lid to prevent contamination.



Figure 1: CRT::LUC concatemer construct. The concatemer contains a minimal -70 promoter (-70 prom), four copies of the CRT/DRE promoter motif (CRT) fused to a luc+ gene (*LUCIFERASE*). Black bars represent forward and reverse primers listed below. Luc+ is a codon-optimised version of the luciferase gene that has been designed to express more efficiently in plants than the original luciferase gene found in fireflies (Whalley et al., 2011).

II.3.2.3 Bombardment

A new 1100psi rupture disc was washed in 100% isopropanol and inserted into a BIO-RAD PDS-1000/He Biolistic® chamber for each bombardment (Figure 2). A stopping screen was placed over each macrocarrier disc in its holder and inserted into the biolistic unit. The seedlings on the agar plate were placed approximately 10cm below the rupture disc holder for bombardment. A 25mm Hg vacuum pressure was applied by a (JAVAL CD0544) double-stage vacuum pump. Once 1100psi was reached using pressurised helium gas, the DNA-coated gold particles were fired at the plants.

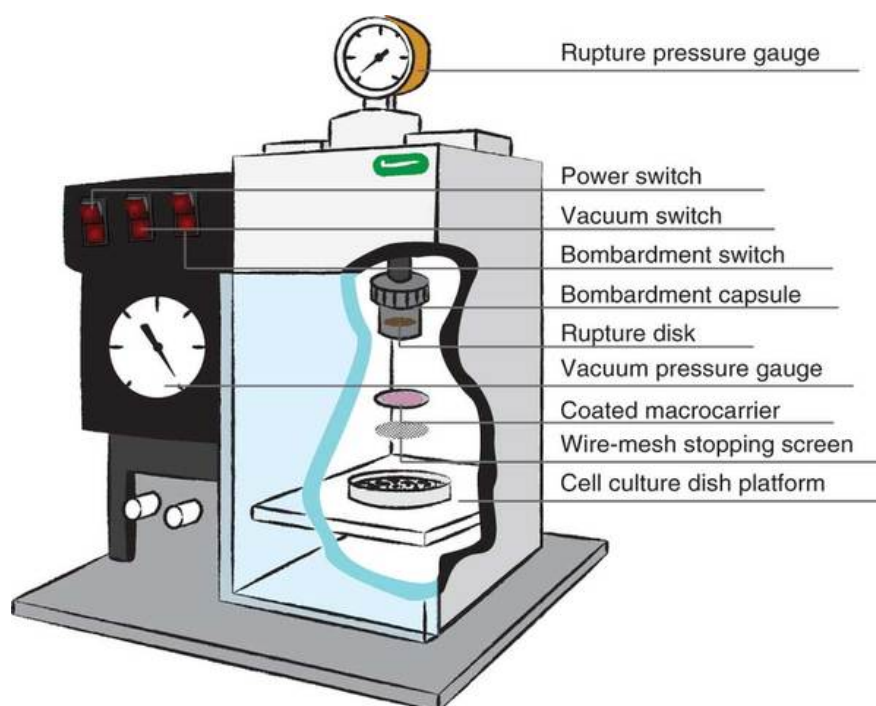


Figure 2: Image from Wu *et al.*, (2012) showing a diagram of the biolistic chamber and equipment used for transient plant transformation.

II.3.2.4 Imaging

The bombarded plants were returned to ambient conditions for 24h and then placed in either cold (5°C) or ambient (20°C) conditioned SANYO growth chambers for 24h to

express the concatemer construct. After 24h, plants were returned to their original growth conditions for 1h to allow translation of the concatemer transcripts. Plants were sprayed evenly with 5mM luciferin in 0.01% Triton X-100 and immediately imaged under an intensified CCD photon counting camera system (Photek 216, Photek Ltd) for 10 minutes. Photon count data was quantified using the Photek 32 image processing software. Tissue was then harvested, frozen in liquid nitrogen and stored at -80°C until use for RNA extraction.

II.3.3 Protoplasts

II.3.3.1 *Isolation*

The photosynthetic tissues of the plants were pre-plasmolysed in a petri dish containing 0.5M mannitol for 1h in the dark. After 1h, the mannitol solution was gently removed and replaced with 30ml of Enzyme Solution and left to digest overnight in the dark at 20°C. The contents of the petri dish were filtered through two successive sieves of 140µm and 70µm respectively. The crude protoplast filtrate was transferred to a 50ml Falcon tube and diluted with 0.5volumes of 200mM CaCl₂. Protoplasts were sedimented at 60xg for 5 minutes in a (Beckman Coulter Allegra™ X-22R) centrifuge at 20°C. The supernatant was carefully removed, protoplasts were gently resuspended in RB I and centrifuged as before. The supernatant was again removed and the protoplasts gently resuspended in RB II. The protoplast suspension was centrifuged as before and the sedimented protoplasts were finally resuspended in 7.5ml W5 solution. Protoplasts were counted using a haemocytometer and resuspended to give 5x10⁶ protoplasts/ml in the mannitol/Mg solution.

II.3.3.2 *Transformation*

50µg of herring sperm DNA at a concentration of 10mg/ml was added to a 15ml Falcon tube containing 300µl of protoplast suspension at a concentration of 5x10⁶ and mixed well. 350µl of PEG-CMS was then added; the solution was carefully mixed to achieve homogeneity and incubated for 30 minutes at room temperature. The protoplast transfection mixture was carefully diluted in a stepwise manner over a 20 minute period. 0.6ml, 1ml, 2ml and finally 4ml of W5 solution was added at 5 minute intervals. The protoplasts were recovered by centrifugation at 600 rpm for 5 minutes and washed in Mannitol/W5 solution. The protoplasts were centrifuged as before and finally resuspended in 3ml Culture Medium to be transferred into 2 1.5ml Eppendorf

tubes. The protoplasts were cultured overnight in the dark to express the construct and RNA was extracted as described below.

II.4 Nucleic Acid Extraction

II.4.1 DNA Extraction

II.4.1.1 *Edward's Extraction*

Based on the Edwards *et al.*, (1991) plant DNA extraction protocol

Approx. 10-20 7 day old seedlings were harvested and placed in an Eppendorf tube before being flash frozen in liquid nitrogen. The frozen tissue was ground in 200-400µl of Edward's Extraction Buffer and centrifuged at 16,000x g for 1 min. $\frac{3}{4}$ volume of the supernatant was transferred to an equal volume of 100% isopropanol. The samples were incubated at 20°C for 5 mins and centrifuged at 16,000xg for 10 mins. The supernatant was removed and the pellet dried in a vacuum desiccator (Eppendorf AG Concentrator 5301). The DNA was resuspended in 50µl TE buffer overnight at 4°C.

II.4.1.2 *Phenol chloroform DNA extraction*

For use in genotyping PCR, DNA extracted from an Edward's prep was diluted one in three in TE buffer (10mM Tris-HCL pH8.0 and 5mM EDTA pH7.5). For ChIP analysis, the DNA was not further diluted in TE buffer.

The DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 ratio, buffered in TE pH 8.0) separated by centrifugation at 16,000xg, the supernatant was removed DNA was extracted again with an equal volume of 100% chloroform. The supernatant was removed and 1/10 of a volume of 3M sodium acetate (pH5.3) and 2.5 volumes of 100% ethanol were added and vortexed. The DNA was precipitated at -80°C for 30 mins and pelleted in a cooled (15°C) centrifuge at 155,000RPM for 30 mins. The pellet was washed with 70% ethanol, dried in a vacuum desiccator (Eppendorf AG Concentrator 5301) and resuspended in the original starting DNA volume of TE buffer.

II.4.1.3 *Mini preps*

Plasmid DNA was extracted using the QIAGEN QIAprep® Miniprep kit and protocol according to the manufacturer's instructions. Briefly, 2ml of a 5ml transformed bacteria culture was harvested by centrifugation and resuspended in a resuspension buffer. Cells were then lysed in a lysis buffer. Protein and genomic DNA were precipitated out

of the solution and the supernatant was passed through a QIAprep spin column. The plasmid DNA was washed on the column and then eluted in 50µl elution buffer. Correct DNA construct was verified by DNA digest. The DNA was quantified using a Nanodrop spectrophotometer (ND-1000 Labtech) and stored at -20°C until use.

II.4.1.4 *Maxi preps*

Total plasmid DNA was extracted using the QIAGEN® Plasmid maxi purification kit and protocol. Briefly, a 200ml culture of transformed bacterial cells were harvested by centrifugation, resuspended in the resuspension buffer and then lysed in a lysis buffer. Proteins and genomic DNA were precipitated out of the sample and the remaining supernatant was passed through an equilibrated DNA-binding QIAGEN-tip 500 column. The retained plasmid DNA was washed on the column and the DNA was eluted in 15ml elution buffer. The eluted DNA was precipitated with isopropanol and washed in 70% ethanol. The DNA pellet was air-dried and finally dissolved in 100µl TE buffer. DNA yield was determined using a Nanodrop Spectrophotometer. Correct DNA construct was verified by DNA digest. The eluted DNA was stored at -20°C until use.

II.4.2 RNA extraction

Total RNA was extracted using the Qiagen RNeasy® Mini RNA extraction kit and protocol. Up to 100mg of plant tissue was harvested and flash frozen in liquid nitrogen. Briefly, the tissue was lysed and homogenised with the QIAshredder column. The flow-through was transferred to an RNA-binding column. The retained nucleic acids were then washed on the column membrane with additional on-column DNase I digestion to eliminate genomic DNA contamination. The column was washed with an 80% ethanol buffer and the RNA was finally eluted in 30µl water for quantification using a Nanodrop Spectrophotometer (Labtech). Eluted RNA was stored at -80°C until use.

II.5 Quantitative Real time PCR

Unless otherwise stated, relative gene expression levels were analysed by quantitative real-time reverse transcription PCR (qRT-PCR) using an Applied Biosystems 7300 system. The reaction was carried out in an optical 96-well plate with three technical replicates for each sample primer pair.

II.5.1 cDNA synthesis

cDNA was synthesised using 2µg RNA per 20µl reaction according to the manufacturer's instructions: reverse transcriptase buffer, random primers, 50µl Multiscribe™ Reverse Transcriptase (Applied Biosystems) and dNTPs (at a final concentration of 4µM) were added to the 2µg RNA. The cDNA synthesis reaction was carried out using the following program (Px0.2 Thermal Cycler): 10mins 25°C, 2h 37°C, 10secs 85°C. The cDNA was stored at -20°C before being diluted 1:50 for use in real-time PCR. Two controls were carried out for each cDNA synthesis reaction; one contained no Multiscribe reverse transcriptase (NRT) enzyme to reveal any genomic DNA contamination, the other contained no RNA template (NTC) for the reaction to identify potential formation of primer dimer products in the subsequent qRT-PCR experiments.

II.5.2 Primer efficiency tests

All primers used for qRT-PCR analysis were designed using the Primer3 program (<http://bioinfo.ut.ee/primer3-0.4.0/>). Transcripts were detected using gene-specific primers at a concentration of 5µM which were first validated for efficient amplification of target DNA using Fast Start SYBR green mastermix with added ROX (Promega) as a passive reference dye. 5µl of 1:10, 1:20, 1:40 and 1:80 diluted cDNA was used in a 15µl reaction mix. Primer regression coefficients showing the efficiency of the primers in amplifying the PCR product can be found in Appendix III. Primer dissociation curves were also analysed for the presence of genomic DNA template amplification and primer dimer formation which may create falsely elevated Ct values in the experiment.

II.5.3 Transcript quantification

Transcripts were detected using Fast Start SYBR green mastermix with added ROX. 5µl of 1:50 diluted cDNA was used in the 15µl reaction. Expression levels of cold-, dark- and sucrose-induced genes were normalised to the expression of *PEX4* (At5g25760) which does not change in response to these stimuli. Expression levels of UV-induced genes were normalised to the expression of a different gene, At4g26410, which does not show an altered expression in response to UV light, as *PEX4* transcript levels are affected by UV light. Relative quantification was performed by the $\Delta\Delta C_T$ (comparative C_T) method as described by Livak & Schmittgen (2001). Error bars represent a 95% confidence interval calculated using a Student's *t*-test and RQ values for each sample were calculated as described by Knight *et al.* (2009). Each primer pair was also tested

with NTC and NRT controls for genomic amplification from the cDNA synthesis and primer dimer formation.

II.6 Genotyping

II.6.1 NASC T-DNA insert lines

Unless otherwise stated, all PCR reactions were carried out using the following program: 5 minutes at 95°C followed by 35 cycles of 30s at 95°C, 30s at 58°C, 45s at 72°C. The final stage consisted of two minutes at 72°C. 5µl of the PCR product was run on a 1% agarose (Bioline BIO-41025) 0.5x TBE electrophoresis gel at 35mA for 1h. Images of the gel were taken using a camera (spacecom 8-48mm lens) mounted on a UV transilluminator (uvitec DOC-CF08.TFT).

T-DNA inserts in NASC seed lines were detected using gene-specific primers designed by the T-DNA express PrimerL program (<http://signal.salk.edu/cgi-bin/tdnaexpress>) and a primer at the left border (LB) of the T-DNA insert at a final concentration of 5µM. 30µM MgCl₂, 0.2µM dNTPs, 1µl of Bio Taq Red was added per reaction and 1µl of DNA was also added in a total reaction volume of 20µl. The amplicon of the T-DNA left border primer (LB) and reverse primer (R) is smaller than the forward (F) and reverse (R) product of the wild-type gene and can therefore be seen as separate distinct bands on an agarose gel (Figure II.3). For each confirmed homozygous seed line acquired from NASC, transcript levels of the knocked-out gene were also investigated by qRT-PCR to ensure transcripts were reduced compared to wild-type.

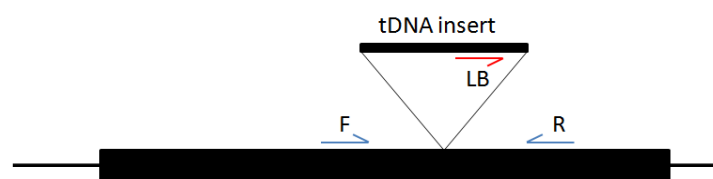


Figure 3: diagram of primer pairs used to genotype NASC tDNA insert plant lines.

II.6.2 EMS *sfr6* genotyping

The *sfr6-1* EMS line contains a single nucleotide polymorphism (SNP) that introduces a premature STOP codon early in the sequence (Knight *et al.*, 2009). As such, the mutation was undetectable using conventional PCR genotyping previously described. A fluorescent probe with specifically-designed primers was therefore used to detect the

single nucleotide polymorphism of the *sfr6-1* plants. The fluorescent Taqman probe binds over the SNP-containing region and uses VIC and FAM as reporter dyes for the two different sequences. Genotyping was carried out according to the manufacturer's instructions for an Applied Biosystems 7300 system. Between the pre-read, amplification and post-read cycles, the 96-well plate was removed, centrifuged for 1 min at 3000 xg to collect the sample at the bottom of each well.

Due to the various inhibitors present in the crude DNA extracted with the Edward's protocol, DNA from putative *sfr6-1* plants was first extracted using the Edward's extraction protocol previously described and then purified using the previously described phenol chloroform extraction protocol. The purified DNA was resuspended in TE for quantification using a Nanodrop Spectrophotometer. To genotype the plants, 10ng/μl of phenol chloroform purified DNA was used in a 15μl reaction. Alternatively, 10ng/μl crude DNA extracted using the Edward's protocol could be used with PerfeCra® qPCR ToughMix™ with added ROX (Quanta 95113-250).

II.6.3 MED15 genotyping

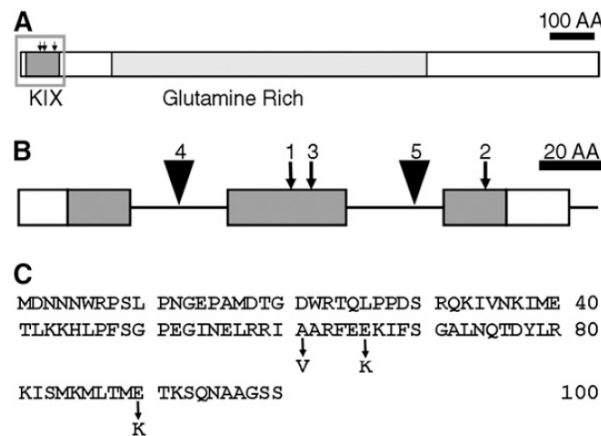


Figure 4: From Canet et al. (2012), (A) Drawing of the predicted NRB4 protein, showing the conserved KIX domain and the region rich in Gln. AA, amino acids. (B) Magnification of the KIX domain, showing the introns (horizontal lines), the point mutations (arrows), and the T-DNA insertions (triangles) found in *NRB4* (At1g15780). The number above the mutation indicates the number of alleles. Only a section of the *NRB4* gene is shown; the region shown corresponds to the gray rectangle in (A). (C) Sequence of the first 100 amino acids of NRB4, indicating the point mutations. *nrb4-4* was SAIL_792_F02, and *nrb4-5* was GABI_955_E02.

Three NRB4/MED15 mutant lines (*nrb4-1*, *nrb4-2* and *nrb4-3*) used in this study were a kind gift from the Tornero group (Figure II.4). Mutant lines were detected by previously

described genotyping PCR and then by restriction digest, with the use of BseX1, Bpi1 and Mbo1 enzymes which cut wild type *NRB4*, *nrb4-2* and *nrb4-3* respectively as the altered amino acids introduced new cleavage sites compared to the wild type *NRB4/MED15* gene.

II.7 Chromatin Immunoprecipitation

II.7.1 Cross-linking

Approximately 5g of seedling tissue was collected. 30ml of Extraction Buffer I containing 1% v/v formaldehyde for protein cross-linking was added to each sample. (At this stage, Extraction Buffer I does not require Triton X-100 or protease inhibitors.) Miracloth was used to keep the seedlings submerged in Extraction Buffer I as a 28mmHg vacuum was applied for 20 minutes (BIO-RAD PDS-1000/He Biolistic®). 2M glycine was added to a final concentration of 200mM to stop the cross-linking reaction. The solution was mixed well and a vacuum was applied as before for 5mins. The tissue was recovered and washed with distilled water to remove the formaldehyde solution, then washed a second time with Extraction Buffer I (formaldehyde, protease inhibitors and Triton X-100 not required). The tissue was dried briefly and transferred to a 50ml Falcon tube before being flash frozen in liquid nitrogen. Tissue was stored at -80°C until use.

II.7.2 Chromatin preparation

The frozen tissue was ground to a fine powder in liquid nitrogen and resuspended in 25mL Extraction Buffer I. The samples were incubated on ice for 5 minutes with gentle mixing and filtered through two layers of miracloth. The filtrate was then centrifuged at 4°C for 15 mins at 3000xg (Beckman Coulter Allegra™ X-22R). The supernatant was discarded and the pellet was gently resuspended by pipetting in 25mL Extraction Buffer I, incubated on ice for 5 minutes and centrifuged as before. Each crude nuclear pellet was resuspended in 1ml Extraction Buffer II, transferred to a microfuge tube and incubated on ice for 5 minutes with intermittent mixing and centrifuged for 10 minutes as before.

II.7.3 DNA fragmentation

The crude nuclear pellet was fully resuspended in 500µl Nuclear Lysis Buffer and incubated on ice with intermittent mixing for 5 minutes. The chromatin was sheared to

achieve an average fragment size of 400bp using a Diagenode Bioruptor (20 cycles at high power of 30s off/15s on).

One aliquot (25 μ l) of the chromatin from each sample was transferred to fresh Eppendorfs to check for digestion efficiency and to quantify the chromatin present in each sample. An equal volume of TE buffer containing 10 μ g.mL⁻¹ RNaseA was added to each sample and incubated at 30°C for 30mins. An equal volume of 20% w/v Chelex (SIGMA C7901-25G, Chelex 100 sodium form) was added. The samples were briefly vortexed and boiled at 95°C in a heating block (Labnet AccuBlockTM Digital Dry Bath) for 10 mins with intermittent mixing. The tubes were returned to room-temperature and then incubated at 50°C for 30mins with 2mg.mL⁻¹ proteinase K (New England BioLabs P81025). The supernatant was recovered by centrifugation at 16,000xg for 1minute. The DNA was extracted using the phenol chloroform extraction protocol described previously. 2 μ l of Linear Polyacrylamide (AppliChem) was added prior to the precipitation step at -80°C. The extracted DNA pellet was dissolved in the original starting volume of TE with 10 μ g.mL⁻¹ RNase A and incubated at 37°C for 30 minutes. The DNA was run on a 1.5% agarose gel to see the size distribution of the fragments. 2 μ l of the DNA was also quantified using a Nanodrop Spectrophotometer (Labtech ND-1000). If fragmentation was to the correct level, 1/10 volume of 10% Triton X-100 was added to the fragmented chromatin and centrifuged at 15,500xg for 10 minutes at 4°C. The supernatant was removed and stored at -80°C until use.

II.7.4 Bead Pre-Clearing

The following steps were carried out at 4°C or on ice. The chromatin was diluted with ChIP Dilution Buffer to achieve a concentration of 25ng. μ l⁻¹ DNA in a final volume of 600 μ l per ChIP reaction.

30 μ l Pierce pre-blocked ChIP grade A/G plus agarose beads (Thermo Fisher, 26159) were prepared to serve 10 μ l per 0.5ml immunoprecipitation reaction plus 20 μ l per sample for pre-clearing. The beads were washed twice with ChIP Dilution Buffer and resuspended in 5 bead volumes of ChIP Dilution Buffer. 20 μ l of the washed beads were added to each sample and incubated on a rocking platform at 4°C for 4-8h to eliminate non-specific binding to the beads during the IP reaction. The pre-clearing beads were removed from the chromatin solution by centrifugation at 3000 xg and retention of the supernatant.

II.7.5 Pre-coating the antibody

2.5µg of Pol II CTD antibody (AbCam ab5408) was added to the pre-cleared ChIP A/G agarose beads and incubated at 4°C on a rocking platform for 4-8h. The antibody-coated beads were recovered by centrifugation and washed three times with ChIP Dilution Buffer. The beads were finally resuspended in sufficient ChIP Dilution Buffer to add 100µl of the bead suspension to each IP reaction.

II.7.6 Immunoprecipitation

The antibody-coated ChIP beads were aliquoted out to give 100µl to each chromatin sample. 500µl of the pre-cleared chromatin was aliquoted into tubes with the antibody-coated ChIP beads and incubated on a roller-mixer platform at 4°C overnight.

The remaining fraction of pre-cleared chromatin was retained as the Input fraction and was flash frozen in liquid nitrogen and stored at -80°C until use.

II.7.7 Washes

The beads were recovered from the IP reactions by centrifugation at 3000xg for 1 minute.

Each wash was carried out in 1.5mL eppendorfs with 1mL of each wash buffer on ice for 5 minutes with intermittent mixing. The beads were recovered after each wash by centrifugation at 3000xg for 1 minute. The beads were washed twice in the Low Salt Wash Buffer, twice in the High Salt Wash Buffer, once with the LiCl Wash buffer and finally twice with TE buffer. After the second TE buffer wash, the beads were resuspended in 50µl TE buffer.

II.7.8 Cross-link reversal

An equal volume of 20% w/v Chelex was added to the bead suspension. 20% Chelex was also added to the Input ChIP fraction. All of the samples were incubated at room temperature with intermittent mixing for 5 minutes. The cross-linking was reversed in a 95°C heating block for 10 minutes with intermittent mixing. Once the tubes had cooled to room temperature, 20µg.µl⁻¹ proteinase K was added and incubated at 50°C for 30 minutes, followed by boiling for 10 minutes. The beads were removed from the samples by centrifugation at 16,000xg for 1 minute. 2 volumes of TE were added onto the beads to recover any traces of IP DNA left and added to the initial supernatant. 5µg RNaseA was added to each sample and incubated at 37°C to 30mins. The DNA was extracted using the phenol chloroform protocol described previously. 2µl of Linear

Polyacrylamide (AppliChem) was added prior to the precipitation step at -80°C. The extracted DNA pellet was dissolved in 200µl TE buffer for use in real-time PCR.

II.7.9 Quantitative real time PCR analysis of ChIP DNA

MgCl₂ was added to a final concentration of 1mM in the real-time PCR reaction mix to counteract the chelating effects of the EDTA in the TE buffer. DNA was stored at -80°C until use. Freeze-thaw cycles should be avoided to minimise DNA degradation. ChIP DNA was normalised against the total input fraction of each sample, diluted to the same concentration. The previously described qRT-PCR method was then followed.

II.8 Microarray data

Col-0 and *sfr6-1* Arabidopsis were grown as previously described for RNA extractions and subjected to either 5°C or 20°C for 3h before harvesting and subsequent analysis on Affymetrix gene chips (GEO reference GSE6167). For the second microarray, Col-0, *sfr6-2* and *sfr6-3* were grown as previously described for RNA extraction and subjected only to 5°C for 3h before harvesting and analysis on Affymetrix gene chips (GEO reference GSE46084). The aim of the microarray experiments was to generate two lists of cold-inducible genes that were and were not mis-regulated in the three different *sfr6* allele mutant lines compared to wild type. Microarray data analysis was carried out using the WindowsTM dChip Programme (Li & Wong 2003) to generate the two lists of cold-upregulated genes that are misregulated and non-misregulated in *sfr6*. Microarray analysis was conducted prior to start of the project. Full details of the analysis can be found in Hemsley *et al.*, 2014.

Analysis of overrepresented heptamer sequences 500bp upstream of the start codon was performed using the “oligo analysis” programme available on the Regulatory Sequence Analysis Tools (RSAT) website (<http://rsat.ulb.ac.be/rsat>). All default settings were used with the exception of “Oligomer length” and “organism”, which were set to 7 and *Arabidopsis thaliana*, respectively (Thomas-Chollier *et al.*, 2011). Matrices from the RSAT programme were entered manually into the Weblogo programme (<http://weblogo.berkeley.edu/logo.cgi>) (Crooks *et al.*, 2004) to graphically represent the nucleic acid multiple sequence alignments. All default settings were used with the Weblogo programme. The Athena program (http://www.bioinformatics2.wsu.edu/cgi-bin/Athens/cgi/analysis_select.pl) was used to explore previously characterised

transcription factor binding sites. All default settings were used with the exception of selecting 500bp upstream of the start codon for analysis.

II.9 Primer sequences

II.9.1 Primers used for genotyping NASC T-DNA insert and EMS lines

Gene Name	AtG code	Forward Primer	Reverse Primer
<i>CYCC-1</i>	At5g48640	5' GATGGGCAATTGTGGAAGTC 3'	5' CCTTCAGCAAATTGGCTTTG3'
<i>CYCC-2</i>	At5g48630	5' GCAATGTAAATGCAAGCGAGT3'	5' AACTTGGGGTCTTGCAACG3'
<i>HEN3/CDK8</i>	At5g63610	5' TTGGTCTTGGCATCGATCTAC3'	5' TTGGTGAAGGCACTTATGGTC3'
<i>NRB4 (MED15)</i>	At1g15780	5'AATCTATGGATGTGCCATTATTAGCG3'	5' TGCAGCAGAATGGAAACTAA3'
<i>SFR6/MED16</i>	At4g04920	5' CGCTGAGAGATTCTGGTGA3'	5' TGAAACTATCCCATCCTCTGC3'
<i>SFR6</i> TaqMan Probe	At4g04920	5' CGTATGATCCAGATGAAGGTCCTT3'	5'GCAGTACAACAGGTTGAACACTTG3'A
<i>MED14</i>	At3g04740	5' TCCAAACAACAAAACCCATC3'	5' TTCACCGTTTATTGTCGGAAC3'
<i>MED2</i>	At1g11760	5' TTTGCGGTATCAAATCGTTTC3'	5' CTCACAAACTCCTCTGCTTG3'
SAIL LB	-	5' GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC3'	
SALK LB	-	5' GCGTGGACCGCTTGCTGCAACT3'	
CRT::LUC concatamer	-	5' GATGTCCACCTCGATATGTG3'	5' GATGTCCACCTCGATATGTG3'

II.9.2 Primers used for transcript analysis

Gene Name	AtG code	Forward Primer	Reverse Primer
<i>PEX4</i>	At5g25760	5' TCATAGCATTGATGGCTCATCCT3'	5' ACCCTCTCACATCACCAGATCTTAG3'
<i>KIN2</i>	At5g15970	5' CAACAGGCGGAAAGAGTAT3'	5'CAACAACAAGTACGATGAGTACGA3'
At3g52740	At3g52740	5' GAAGCATCGGAGAGAGATCG3'	5' AGCAGTACGTGCAGACGAGA3'
At1g20030	At1g20030	5' TTTGCTCCCTGAAAGAAGAA3'	5' CCTTGACATAACTCCGGAGAAG3'
At1g20030 non-spliced	At1g20030	5' AAGAATCTGCCTGATCTCACA3'	5' TGAAGCTCCTTGACATAACTC3'
At1g68500	At1g68500	5' AGCTCTGTGTGGTTGCCTT3'	5' CGTCCCAACAACATCATTG3'
At5g62360	At5g62360	5' AGCATGCTCAAATTGGTTC3'	5' TGGGTACGTTGTGAAAGTGC3'
At5g46710	At5g46710	5' CCATGAAACTTGCATTGAGC3'	5' CGAGAACCCAAATCGAGAAA3'
<i>ABF1</i>	At1g49720	5'AATGAAAACCCATAATAGTGAGGTAA3'	5' ATTGTCTTTTGGCCAGCAAT3'
<i>SZF2</i>	At2g40140	5' TCAAAAACCCCAACCACTTC3'	5' TGCACCGCACATATCTCTTC3'
<i>LT178</i>	At5g52310	5' GCACCCAGAAGAAGTTGAACA3'	5' TCATGCTCATTGCTTTGTCC3'
<i>COR15A</i>	AT2G42540	5' CGTTGATCTACGCCGCTAAAG3'	5' CTCACCATCTGCTAATGCC3'
<i>COR414</i>	AT1G29395	5' GGGAGAGTATGGTGTATGGGCA3'	5' TGATATGGGCCACAATCA3'
<i>DIN6</i>	At3g47340	5' GGCCAAGAGAGTTCGTGTTTC3'	5' AGACGTTGATGGGCCAAGTA3'
<i>MED14</i>	At3g04740	5' TAGCTTTGGTTCAAGCGTTT3'	5' GGCCATAGTTGGTCAGGAGA3'
<i>MED2 (MED32)</i>	At1g11760	5' GCGACTAGTCTCCCTCCCAT3'	5' CCATCGAACCCTCTACTCATC3'
<i>PR1</i>	At2g14610	5' CATCCTGCATATGATGCTCCT3'	5' TCGTGGGAATTATGTGAACG3'
At4g26410	At4g26410	5' CTCGTTCCCTCCGTGAAAT3'	5'TGAAGAAAGCATTCTCATAGGTCTT3'

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At1g76590	At1g76590	5' GTTCTGCTCTCTCGGTTGC3'	5' CGCTTCATCAGATCCCACT3'
At1g27730	At1g27730	5' GGCATAGGGCTCATGACTTC3'	5' GGTCCACTAGCCACGTTAGC3'
At1g32860	At1g32860	5' ATGCTCACCACCAATGACAA3'	5' GGATAGTGCGTCCACTGGTT3'
At5g46710	At5g46710	5' CCATGAAACTTGCAATTGAGC3'	5' CGAGAACCCAAATCGAGAAA3'
At5g48250	At5g48250	5' ATTGCTTAATGCTGGCTGCT3'	5' TTGGTTCAGCCTTCAACTCA3'
At2g45660	At2g45660	5' AACCGGTTTGGTGCTGACT3'	5' AACTTTATGAATTCGCCAGCTC3'
At1g53035	At1g53035	5' TGCAACAGAAAGCAAACCAG3'	5' GGACCAGGAAGTGAGATTGC3'
At1g20030	At1g20030	5' TTTGCTCCCTGAAGAAGAA3'	5' CCTTGACATAACTCCGAGAGA3'
At2g36220	At2g36220	5' AGTCCCAGGGGAAAGTAGT3'	5' CACGTCATCACCAACGACTT3'
At3g04010	At3g04010	5' CCGTTGTACGCTGTCAAGAA3'	5' AGATTGGGTCCAAAGAAACG3'
<i>ERF5</i>	At5g4723	5' TCTTCGGATCATCGTCTCTTC3'	5' GGTTCGATACGGATTCAGAGAA3'
At2g39920	At2g39920	5' CGAATACCGACTCCCAAT3'	5' ATGTCGGCTTATACGCATCC3'
At1g68500	At1g68500	5' AGCTCTGTGTGGTTGCCTT3'	5' CGTCCCAAACAACATCATTG3'
At3g25870	At3g25870	5' CTA AAAACATTTTGATTTTCTAATCC3'	5' CGTACCACCGTTTCACTAT3'
At1g48100	At1g48100	5' CGAAGGCAATGGTCAAAAAT3'	5' GTCGGACTTGACATGGTC3'
At5g62360	At5g62360	5' AGCCATGCTCAAATTGGTTC3'	5' TGGGTACGTTGTGAAAGTGC3'
At4g24960	At4g24960	5' CCAACATCCTCCACTCCATT3'	5' CCACCACCAAGACCATTACA3'
<i>GOLS3</i>	At1g09350	5' GAGGTTACAGGCCAAGAAG3'	5' TCGTTGTAATGTCCCACCAT3'
At3g13310	At3g13310	5' TACCCGTCTCCGTGGTACTC3'	5' TCACAAAGCTTACGCGACAC3'
At3g17130	At3g17130	5' GCGTTTCGTGATTCCTTTCTG3'	5' AATATTCCTCAGGCGATGGA3'
At3g61190	At3g61190	5' CTCTAATCTCGGCTCCACA3'	5' TTAATCGGATCCCACCAGA3'
<i>LT178</i>	At5g52310	5' GCACCCAGAAGAAGTTGAACA3'	5' TCATGCTCATTGCTTTGTCC3'
At2G47990	At2G47990	5' CAGCGTCAAGGTATGGGATT3'	5' TCATGTATCCGTCCAAAGCA3'
At5G66985	At5G66985	5' TATCGTCTGAAACGCAAACG3'	5' AGACATCGCCGCTATCAATC3'
At3g52740	At3g52740	5' GAAGCATCGGAGAGAGATCG3'	5' AGCAGTACGTGCAGACGAGA3'
At1g04040	At1g04040	5' TCTAAGTGTGACGGCATGGA3'	5' TCTTCATGTGTGGCACTGCT3'
At5g41010	At5g41010	5' CACTTTGAAGTCTGGGGATG3'	5' TCTCAGCGAGCTTCGTATTG3'
At5g20180	At5g20180	5' AAACGTCGTGGACGTGTGTA3'	5' ACTAGCAATCGGCTCAGCAA3'
At5g52820	At5g52820	5' GCACACACTTGCTGTGACTTG3'	5' CCCCTGAGTAGTCTCCACA3'
<i>SZF2/CZF1</i>	At2g40140	5' TCAAAAACCCCAACCACTTC3'	5' TGCACCGCACATATCTCTTC3'
<i>POP1</i>	At5g44110	5' CCTCCAACCATATGTTTTCA3'	5' ATCTCGTTGCCTCTTGGTTG3'
At1g52280	At1g52280	5' TTGTCCAGCAGTATCCCAA3'	5' GCTACGATTGGAGCAGATTTT3'T
At5g20600	At5g20600	5' ACGGCCTAAGCTCTTCAACA3'	5' TCGACGGGACTTTACTTGCT3'
<i>JAZ1</i>	At1g19180	5' ATGCAAGCCTGATGTCAATG3'	5' GCTGACGTGAGTTGCCTAAA3'
At1g51610	At1g51610	5' GGGCGAAACAAGTGAAGAGA3'	5' CACGAGGAAGTTGCACCATA3'
At4g02330	At4g02330	5' CGGTTGGACCACTTTCAACT3'	5' AGCGGTATTTGCGAAAGTCA3'
At3g19680	At3g19680	5' AGAAACTCCAACCTCCGTGA3'	5' CGGTTGTTCTTTTCCGAAT3'
At3g02250	At3g02250	5' TCGAGTATCGGTCTTGTTCAGA3'	5' ACCACACCATGCCTCCTATT3'
<i>ABF1</i>	At1g49720	5' AATGAAAACCCATAATAGTGAGGTAA3'	5' ATTGTCTTTTGGCCAGCAAT3'
At3G05800	At3G05800	5' ATCGAATCCTTCCACGTCAC3'	5' GACGTAGAGCCTCCATCAGC3'

II.9.3 Primers used for ChIP analysis

Gene name/position	AGI code	forward primer	reverse primer
<i>PEX4</i> Mid	At5g25760	5' CTTGGACGCTTCAGTCTGTG3'	5' GAACGCCCATGGTTACATTT3'
<i>GOLS3</i> -500	At1g09350	5' TGCATATTTTACCAAAACAAAA3'	5' ATTTTCTTCTTTTGCTAACG3'TC
<i>GOLS3</i> TSS	At1g09350	5' CCATAATCACGGCCTCAAAG3'	5' TCATCTCAGGTGCCATCTTG3'
<i>GOLS3</i> Stall	At1g09350	5' GGAACCGGAGACTACGTGAA3'	5' GCCTTGGTCCAATAGCTGTC3'
<i>GOLS3</i> Mid	At1g09350	5' TGGGGCAGCTACCACTATACA3'	5' GAGCATGGCCAAGACAAGAT3'
<i>GOLS3</i> End	At1g09350	5' TTCACAGGAAACCGAAAACC3'	5' ACAAGAACCTCGCTCGTCAG3'
<i>KIN2</i> -500	At5g15970	5' ATGAAAATACGGGAGGTTGCG3'	5' GTTGTTGCATATGCGTTTGG3'
<i>KIN2</i> TSS	At5g15970	5' GATTACACGTGGCACCACAC3'	5' GGAAGGCATTCTGTGGTGC3'
<i>KIN2</i> Stall	At5g15970	5' GCTGGCAAAGCTGAGGTACT3'	5' CCATGGTCAACACAAAATCAA3'
<i>KIN2</i> End	At5g15970	5' AGTATATCGGATGCGGCAGT3'	5' TCCCAAATTTTATTTGAAAATCC3'
At5g54470 -500	At5g54470	5' GACCGGAACGAGAGAGGAAT3'	5' TCCTACGTGGACGAATCAGG3'
At5g54470 TSS	At5g54470	5' AGGAAGCTTCTTGCATTCA3'	5' TTCTGCGGACACGAATAAGA3'
At5g54470 Stall	At5g54470	5' AGCTTGTGGTTGTGGTTGTG3'	5' GTGTTTCGCCACCAGAAAAT3'
At5g54470 Mid	At5g54470	5' GGTGGTGAAAAGGACGAGAC3'	5' TTGGTTCTCCTCTGCATT3'
At5g54470 End	At5g54470	5' CCTCATCGTTCATTTACTAACAACA3'	5' TGAACAACTTTGCTTCATTTATTTT3'
At3g52740 -500	At3g52740	5' AGTTGAGTTTGGTCACACAAAAA3'	5' AACCTAAAATTCTATCAAGTGATCAAG3'
At3g52740 TSS	At3g52740	5' CGTCGACGTGTAAGACAACC3'	5' TTGCAGGAAACAAAAGATGAA3'
At3g52740 Stall	At3g52740	5' TGATGAACATCGACGATACGA3'	5' GCTTCGGTTTCTCTCAAGC3'
At3g52740 End	At3g52740	5' TCGTCTGCAGTACTGCTCT3'	5' TTGAGGAACAAGAACCACATTT3'
At5g03230 -500	At5g03230	5' CAAAGACGAAGATAAAAAATTCATT3'	5' TGTGGCAAGTCTCACGTTCT3'
At5g03230 TSS	At5g03230	5' AAGTTTAACCAATGAGAGAGCTT3'	5' TCTGGGTTTTGATCAGCTTG3'
At5g03230 Stall	At5g03230	5' CCAGAGATGTCCGATGAGAA3'	5' TCGTCGGTTTTCTCCGTAAC3'
At5g03230 Mid	At5g03230	5' TGGGAGAAGATTGGGTTTCA3'	5' ATAACGCCAAAAGGAAATCG3'
<i>PEX4</i> pro	At5g25760	5' GCAGAGTCATTGCTTAACCTAA3'	5' CCCAAAGAGAACGAATTGTCA3'
<i>GOLS3</i> 3IGR	At1g09350	5' TGCAATTGGTAAGTTCTTCATTTT3'	5' TTTTAGAAGAGATTGTGTGTTGCAT3'
At3g52740 3IGR	At3g52740	5' TGCAATGTGTCCGCTCTAAA3'	5' TAGGAAAACGGGCAAATGTC3'
<i>KIN2</i> 3IGR	At5g15970	5' CGAAATCGTTGTGGTCAATG3'	5' ACAATGACGCAGAGCAAACA3'
At1G20030 Mid	At1g20030	5' GAATAGCGTCGACGGGAAG3'	5' GTAGCTATAGGCGCGTGGAC3'
At1G20030 End	At1g20030	5' TGTGGTGGTTCTTCTGACCA3'	5' CAACGTTTGGTCATGTGGAGT3'
At1G20030 3'IGR	At1g20030	5' ACATGGGCTATTCTCAACG3'	5' TTAGGCGCTGGAGGAGTTC3'
At1G20030 TSS2	At1g20030	5' CATGAGAAAAAGAAAAAGAAATCTCG3'	5' AAGAAAAGAGAGAATAAACATTAGCC3'
At1G20030 -250	At1g20030	5' AATGGTTTCTTAGGACACTTGAA3'	5' TTTTATGGCTTGGAGTTGGA3'
<i>PEX4</i>	At5g25760	5' TGTTTTGTTGCAAATCTCTGTG3'	5' CCTTTGATAAGAGCGGTCCA3'
At3g05800 End	At3g05800	5' ACTAATCTCGGCTCGGCTTT3'	5' TTCGTCCAACCTCGTATGA3'
<i>ABF1</i> MID	At1g49720	5' CAGGCTTATACCTTGGAACTGG3'	5' TTCAGCCTGCAACATAGAGAGA3'
At5g46710 MID	At5g46710	5' TGCCTGCATGGTCTGTTATC3'	5' CAAGGGAGGTAATGGATGCT3'

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At1g68500 End	At1g68500	5' TCGGATCATAGTTCCTTTCT3'	5' GAGGCACGGCGTCTATAAAA3'
At5g62360 MID	At5g62360	5' TGTCAGCTAAGTGGCTCGAA3'	5' ACCTTAACCTTCCCGTCCAT3'
At5g48250MID	At5g48250	5' AGCAGCCAGCATTAAGCAAT3'	5' TTCTCCAGTGATGCCAGAGA3'
<i>COR15a</i> MID	At2g42540	5' CAGATGGTGAGAAAGCGAAAG3'	5' CTTGTTTGCGGCTTCTTTTC3'
<i>LT178</i> MID	At2g42540	5' GACTCCGGTCAATGAGAAGG3'	5' CCGCCACATAATCTCTACCC3'
<i>SZF2/CZF1</i> MID	At2g40140	5' TTCTGCAATGGTTTCACCAA3'	5' TGTTCCTGCCATAAACCA3'

III. Results

III.1. Microarray data

A microarray experiment was previously carried out by the Knight group to investigate genes in wild type and *sfr6*-1 (Warren *et al.*, 1996) *Arabidopsis* that were upregulated in response to cold conditions (6h at 5°C) compared to ambient conditions (6h at 20°C). A second microarray was then conducted to investigate cold-induced gene expression in *sfr6*-2 and *sfr6*-3 mutants in cold conditions compared to wild type. The two microarrays were used to investigate whether these cold-inducible genes were misregulated in three different *sfr6* alleles (*sfr6*-1 is an EMS point mutation that causes a premature stop codon early in the coding sequence, *sfr6*-2 and *sfr6*-3 alleles are T-DNA insert lines) in cold conditions as *sfr6* plants fail to cold acclimate and cannot therefore survive freezing temperatures. Genes were said to be cold-inducible if they showed a minimum 1.5-fold induction in the cold-treated wild-type plants compared to ambient-treated. Cold-inducible genes were said to be misregulated in *sfr6* if they showed a minimum 30% reduction of expression in all three of the *sfr6* mutant alleles compared to wild type levels in the cold. 81 genes from the microarray were therefore said to be cold-inducible and misregulated in all three *sfr6* alleles, based on these criteria (a list of the 81 misregulated genes can be found in Appendix II). For comparison, a second list of 81 genes said to be non-misregulated was created from genes that had the most similar expression values in wild type and all three *sfr6* mutant alleles. A list of these genes can be found in Appendix II)

III.1.1. RSAT

III.1.1.1. 81 misregulated genes contain known cold-inducible transcription factor binding motifs

The RSAT program was used to analyse 500 bp upstream of the transcriptional start site for heptamer sequences that occur at a higher frequency than would normally be expected to occur randomly in the genome. The WebLogo 3 (<http://weblogo.threeplusone.com/>) program was used to graphically represent the promoter motif sequences obtained from the RSAT analysis.

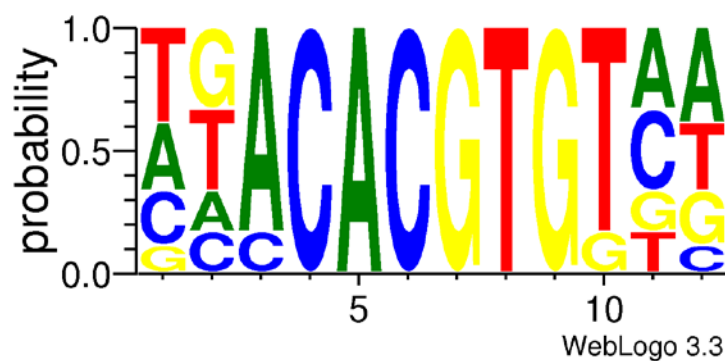


Figure 1: Consensus sequence that contains the abscisic acid response element (ABRE) CACGTG motif was found using the RSAT promoter motif program.

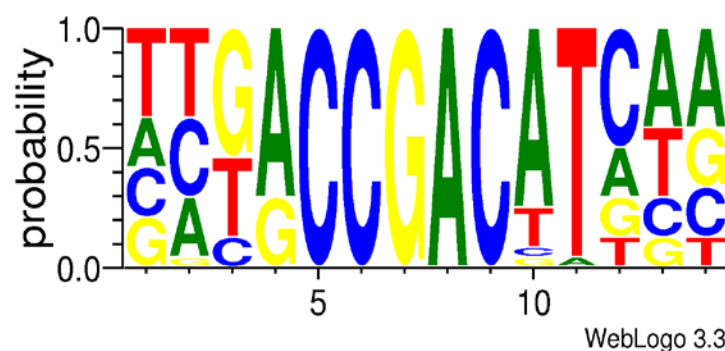


Figure 2: Consensus sequence that contains the C-Repeat/Drought Response Element (CRT/DRE) CCGAC motif was found using the RSAT promoter motif program.

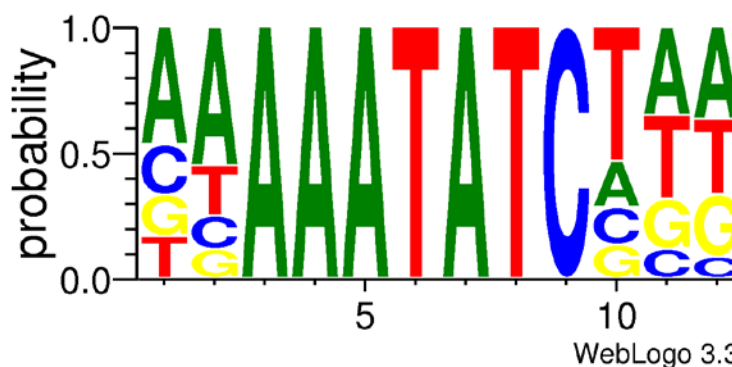


Figure 3: Consensus sequence found that contains the AAATATC sequence which encodes the Sugar Response Element (SRE) promoter motif using the RSAT promoter motif program.

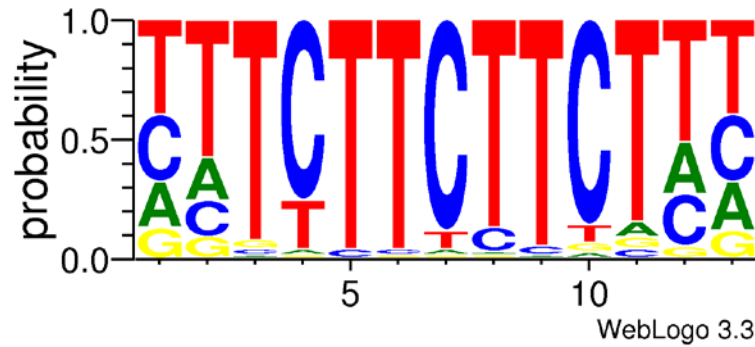


Figure 4: Consensus sequence found in almost all of the 81 misregulated gene promoters using the RSAT promoter motif program. It is not a known binding site for transcription factors in plants.

The CACGTG sequence (Figure III.1) represents the abscisic acid response element (ABRE) promoter motif (Mundy *et al.*, 1990). The CACGTG motif was found at 18 different sites in the 81 misregulated genes. It had a frequency of 0.22 per promoter. The CCGAC sequence (Figure III.2) contains the CRT/DRE promoter motif, found at 24 sites in the 81 misregulated genes and is the binding site of CBF/DREB2 transcription factors (Stockinger *et al.*, 1997, Liu *et al.*, 1998). It had a frequency of 0.30 per promoter compared to the expected significance of 2.5×10^{-13} as shown by Athena analysis software. The ABRE and CRT/DRE elements are previously known to be found in *Cold On-Regulated (COR)* genes. The SRE promoter motif (AAATATC) in Figure III.3 was found in genes that are misregulated in response to cold treatment in *sfr6* and also in previous studies of sugar-responsive genes (Tatematsu *et al.*, 2005). No known transcription factors have been shown to bind to this sequence in plants. The TCTTCTTCT sequence had a frequency of 0.97 per promoter in the 81 misregulated genes (Figure III.4). It is not a known binding site for transcription factors in plants but is known as a Y-Patch-Like motif. A Y-patch motif is a pyrimidine-rich region of DNA and is a direction-sensitive regulatory motif much like the TATA box that plays a major role in transcriptional processes in plants as it is involved in transcriptional regulation by DNA packing and histone binding. It is found close to the transcriptional start site of genes (Yamamoto *et al.*, 2007). A CACGCC sequence was also found using the RSAT software which encodes the low-temperature response element (LTRE) that plays a similar role to the CRT/DRE element in response to low temperature (Medina *et al.*, 1999)

III.1.1.2. 81 non-misregulated genes contain no known transcription factor binding motifs

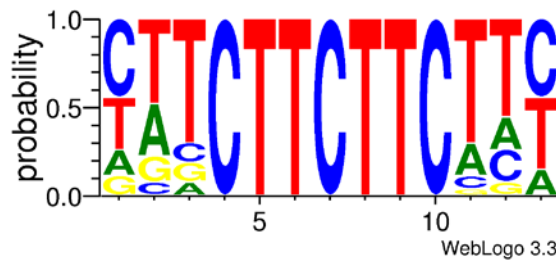


Figure 5: Consensus sequence that contains the Y-patch motif (CTTCTTC) was found using the RSAT promoter motif program.

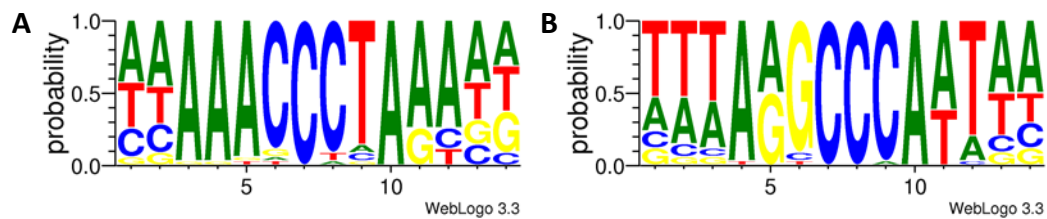


Figure 6: Other sequences also found in the RSAT promoter analysis of the 81 non-misregulated genes.

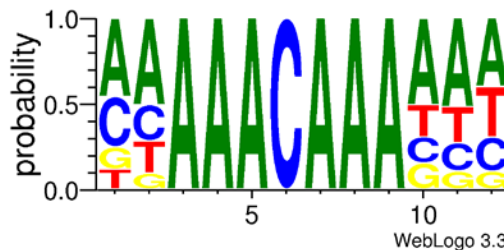


Figure 7: Consensus sequence that contains an AAACAAA motif using the RSAT promoter analysis program.

Fewer consensus promoter motif sequences were found in the 81 non misregulated genes compared to the 81 misregulated genes. The Y-patch motif (Figure III.5) found in the 81 non-misregulated genes had a frequency of 0.33 per promoter. Other motifs were also found that contain AAACCCTA and GCCCA sequences (Figure III.6 A & B) which are not known transcription factor binding sites in plants or involved in transcriptional regulation by DNA packaging. The AAACAAA sequence (Figure III.7) has been hypothesised to be involved in activation of anaerobic genes and is found in sucrose synthase genes. They are not known transcription factor binding sites in plants but are found in animals (Mohanty *et al.*, 2005). Many sequences found by the RSAT software are not yet recognised transcription factor binding sites in plants, but have been identified in previous studies (Mohanty *et al.*, 2005).

III.1.2. Promoter analysis using ATHENA software

The ATHENA analysis software (<http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl>) was used to identify known transcription factor binding motifs that were enriched in groups of gene promoters compared to the expected occurrence of a particular sequence in the plant genome. The software identified the ABRE (CACGTG) and CRT/DRE (CCGAC) promoter motifs to be overrepresented compared to the expected occurrence of the motif in the group of 81 misregulated genes. Athena also identified the full EVENING ELEMENT motif (AAAATATCA), the core sequence of which is equivalent to the SRE in sequence (AAATATC), the full EE has been shown to be present in genes regulated by the circadian clock in Arabidopsis (Harmer et al., 2000). The AAAATATCA motif occurred at 60 sites in the misregulated genes, with a frequency of 0.74 per promoter and is a known binding site for LHY and CCA1 transcription factors that are components of the circadian clock signalling network (Alabadí et al., 2001, Harmer et al., 2000). The ATHENA program did not show the genes to be enriched in any other known motifs. No known plant promoter elements were found in the 81 non misregulated genes using the ATHENA software.

III.2. Monitoring expression of cold-inducible genes chosen from the microarray data

Quantitative reverse transcriptase PCR (qRT-PCR) analysis was carried out on cold-inducible genes chosen from the microarray data to assess if relative expression levels between wild type and *sfr6* mutant plants were similar to those seen in the microarray experiments. Genes were classified into three different groups depending on the previously described cold-inducible promoter elements found within 500 bp upstream of the predicted transcriptional start site. Genes were divided into three groups: those that contained the CRT/DRE element (CCGAC), those that contained the full EE motif (AAAATATCA) and genes that contained both CRT/DRE and EE motifs.

III.3. Assessment of cold-induced transcript levels after various lengths of time in the cold.

A time course experiment was carried out to investigate the kinetics of *COR* gene induction in seedlings under constant light conditions. This was in order to investigate whether these varied depending on the promoter motifs found present within 500 bp

upstream of the transcriptional start site and to assess an optimum point at which to measure differences in *COR* gene expression between wild type and mutants in response to cold (5°C) temperatures. This experiment was also used to investigate ambient gene expression in the free-running clock in plants to see if expression patterns of cold-inducible genes that contain EE promoter element are more strongly up-regulated in the relative evening period compared to the rest of the day.

Unless otherwise stated, all gene expression is normalised to the expression of *PEX4* (Peroxisomal ubiquitin conjugating enzyme 4), a housekeeping gene that does not alter its expression in response to cold temperatures (Czechowski *et al.*, 2005). Graphs show relative quantitation (RQ value) of gene expression compared to the first temperature-treated sample on the histogram (set to a value of 1), as described by the $\Delta\Delta C^T$ method (Livak & Schmittgen 2001) of quantitation. Patterns of gene induction in response to cold (5°C) were consistent across two biological replicate experiments, however slight variation was observed in the degree of gene induction between experiments. Unless otherwise stated, data from a representative experiment of each gene has been shown.

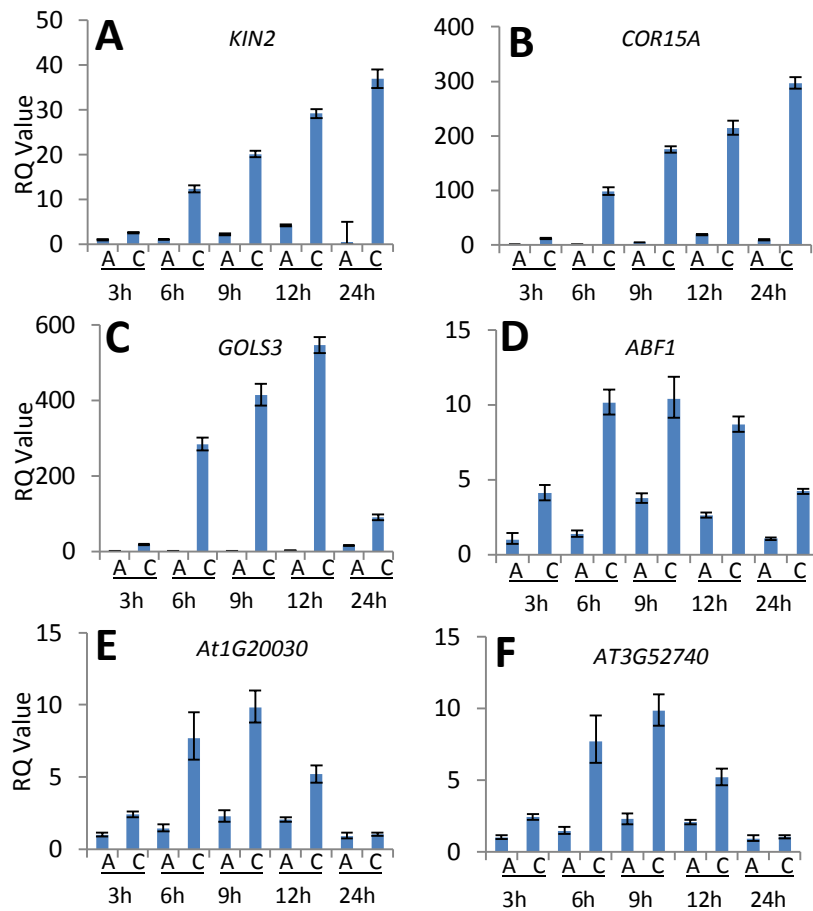


Figure 8: Relative quantification (RQ value) of cold-inducible transcript levels in wild type plants after 3, 6, 9, 12 and 24h in cold or ambient conditions. A: Ambient (20°C); C: Cold (5°C). Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

Genes whose promoter contains the EE (Figure III.8 D & E: *ABF1* & *AT1G20030*) show increased expression under ambient conditions in constant light as the time elapsed since presumptive dawn progresses with the free-running clock. They showed a maximum induction in the cold after 9h before expression starts to decline. CRT/DRE-containing genes (Figure III.8 A, B & C: *KIN2*, *GOL53*, *COR15a*) did not show an increase in transcript levels in ambient conditions, but continued to increase in expression over time in the cold up to 24h. *GOL53* is the exception in the CRT/DRE gene group, which showed a pattern of gene induction similar to genes that contain an EE as it showed reduced expression at 24h compared to 12h. *AT3G52740* (Figure III.8F) is a gene that contains neither CRT/DRE nor EE promoter motifs (selected from the group of 81 non misregulated genes) and showed a pattern of gene expression remarkably similar to the EE-containing *ABF1* and *At1G20030* (Figure III.8D & E) genes during the time

course experiment. These data suggest that 6h at 5°C was a suitable time point at which to assess the expression of genes from all three groups.

III.4. Transcript levels in T-DNA insertional mutant lines

All insertion lines used in this study were obtained from the Nottingham Arabidopsis Stock Centre (NASC). T-DNA insertion lines from NASC that have not been previously published (*MED2*, *MED14*, *CDK8*, *CYCc1* & *CYCc2*) were tested for disruption of the gene by both conventional PCR and qRT-PCR. This was to show the presence of the T-DNA insert and that transcription of the gene under non-experimental conditions was reduced in the proposed mutant line compared to the wild-type.

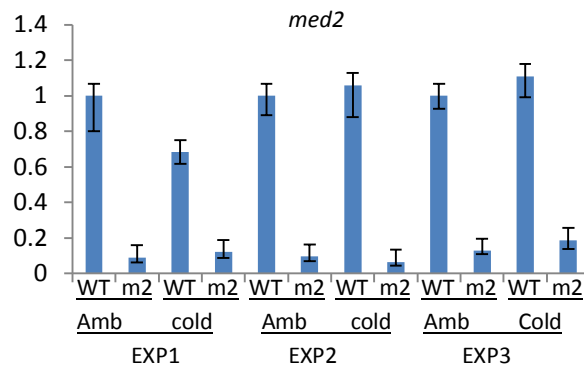


Figure 9: Relative quantification of transcript levels of *MED2* under control and experimental conditions in wild type and *med2-1* lines. Amb: 6h at 20°C; Cold: 6h at 5°C. WT: wild-type; m2: *med2-1* T-DNA insert line (SALK_028490). Error bars represent a 95% confidence interval from 3 technical repeats. Data is from 3 biological replicate experiments.

The SALK_028490 line contains a T-DNA insertion in the promoter region rather than the exon. Data from qRT-PCR experiments in Figure III.9 (above) showed that transcript levels of *MED2* (At1g11760) were reduced by at least 80% in the insertion line under both normal and experimental conditions in three independent experiments of seedlings under cold and ambient conditions. This confirms that the *med2-1* T-DNA insert mutant line used in the subsequent experiments can be considered a loss-of-function mutant. The *med14-1* (At3g04740) insert line used in this study (SAIL_373_C07) contains a T-DNA insert in the last exon and showed reduced expression of full length *MED14* transcript (Zhang *et al.*, 2013).

III.5. Cold-inducible expression of *COR* genes in wild type, *sfr6-1*, *med2-1* & *med14-1* Arabidopsis mutants

III.5.1 Expression of cold-inducible genes

Genes whose promoters contained an EE, a CRT/DRE or both sequences were chosen from the 81 cold-inducible genes misregulated in *sfr6* identified from the microarray. Expression of these genes was tested by qRT-PCR after 6h under cold or ambient conditions in wild type and *sfr6-1* plant lines. This was to validate the differences seen in the microarray data and the parameters used to select the 81 misregulated genes. Expression of these cold-inducible genes was also investigated in *med2-1* and *med14-1* seedlings to examine the effect loss of Mediator tail subunits had on the expression of genes containing different promoter motifs in response to cold.

A 6h time point was chosen based on data obtained from previous time course experiments (Figure III.8). Expression of cold-inducible genes in *med2-1*-and *med14-1* plant lines was also investigated to see if genes were impaired when the MED2 or MED14 subunits were lost from the Mediator complex.

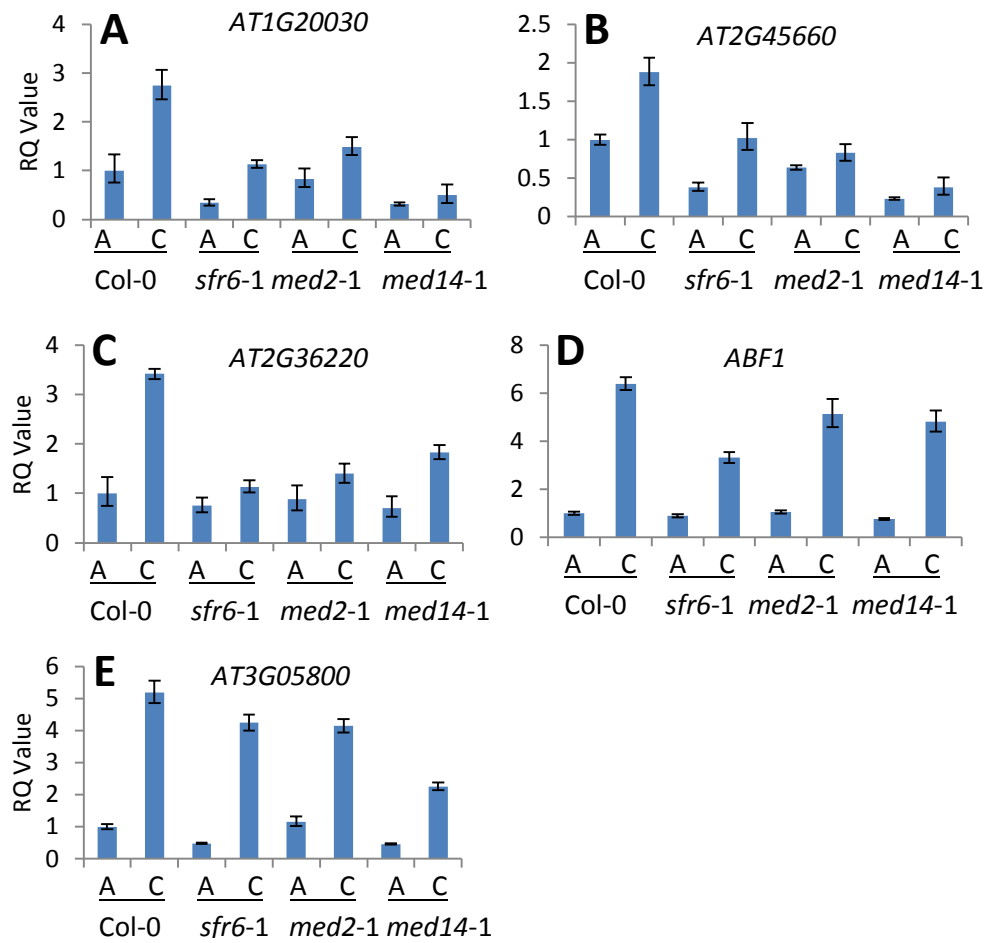
III.5.1.1. *EE-containing genes*

Figure 10: Relative quantification (RQ value) of cold-inducible transcript levels of genes that contain EVENING ELEMENT (EE) promoter elements under ambient and cold conditions. A: Ambient (20°C) C: cold (5°C). Gene expression was measured in response to 6h at 5°C or 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

III.5.1.2. CRT/DRE-containing genes

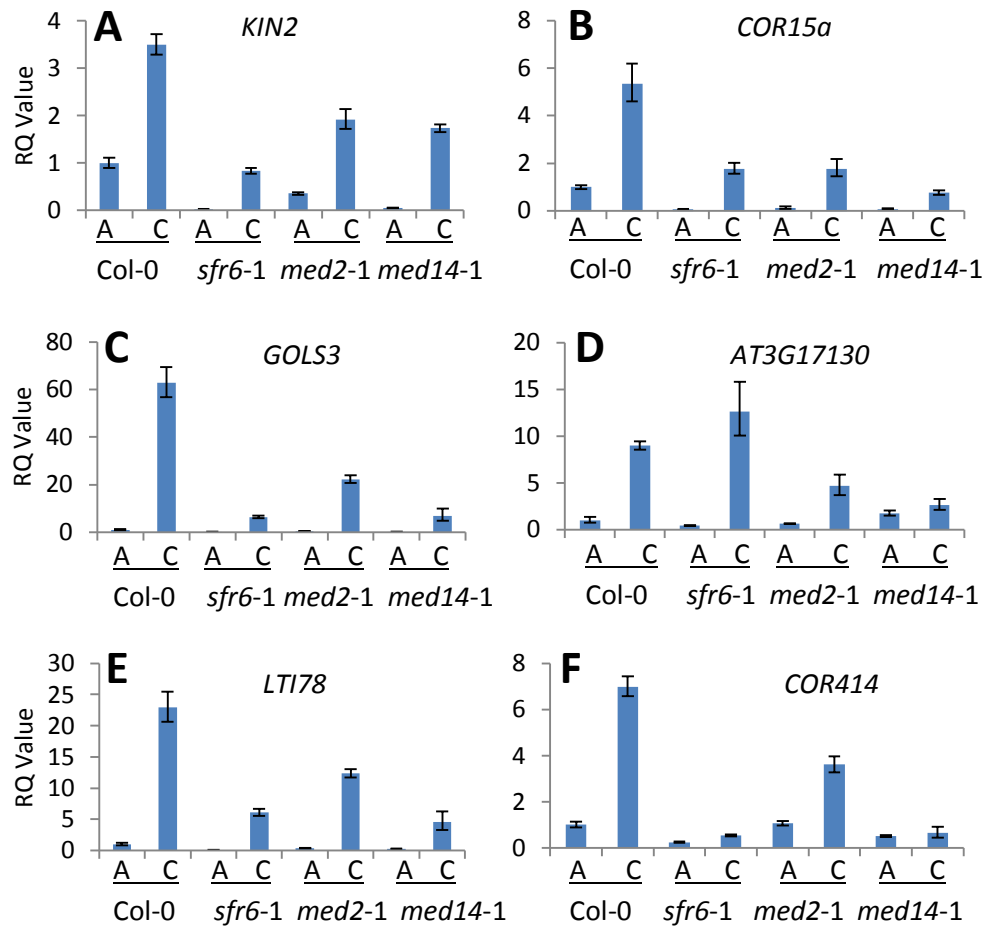


Figure 11: Relative quantification (RQ value) of cold-inducible transcript levels of genes that contain C-repeat (CRT/DRE) elements under ambient and cold conditions. A: Ambient (20°C) C: cold (5°C). Genes are induced in response to 6h at 5°C or 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

III.5.1.3. Genes that contain both CRT/DRE & EE elements

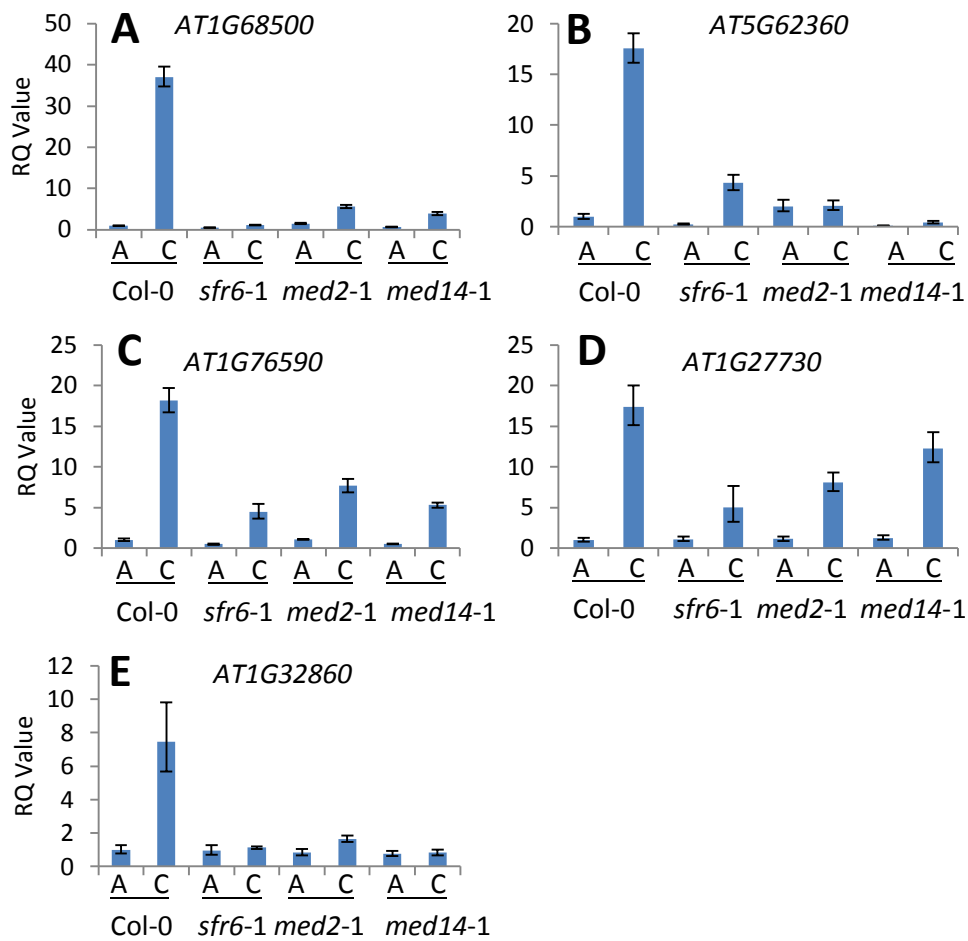


Figure 12: Relative quantification (RQ value) of cold-inducible transcript levels of genes that contain both EVENING ELEMENT (EE) and C-repeat (CRT/DRE) promoter elements under ambient and cold conditions. A: Ambient (20°C) C: cold (5°C). Genes are induced in response to 6h at 5°C or 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments

Data from qRT-PCR experiments (Figure III.10, 11 & 12) show that genes from all three promoter element groups were induced in response to cold (5°C) temperatures in wild type plants compared to the ambient (20°C) control. Data showed that these genes were misregulated in *sfr6-1* seedlings as suggested by the microarray data. These genes were also shown to be misregulated in *med2-1* and *med14-1* seedlings.

COR414 (At1g29395) is a known *COR* gene (Oakenfull *et al.*, 2013) that contains a CRT/DRE motif. However, it was not found in the group of 81 misregulated genes found in the microarray despite qRT-PCR evidence to suggest that it is both cold-inducible and misregulated in *sfr6-1* to a similar degree as other CRT/DRE-containing genes (Figure III.11F).

III.5.1.4. Cold-inducible genes not misregulated in *sfr6* mutants

Genes were chosen from the group of 81 non-misregulated genes to further validate microarray data suggesting that the genes are both cold-inducible and are not misregulated in *sfr6* mutants. Genes were tested for their expression after 6h under cold or ambient conditions in wild type and *sfr6-1*. Expression of cold-inducible genes in *med2-1* and *med14-1* plant lines was also investigated to see if genes were impaired when the MED2 or MED14 subunits were lost from the Mediator complex. The 6h time point was chosen based on data obtained from time course experiments (Figure III.8).

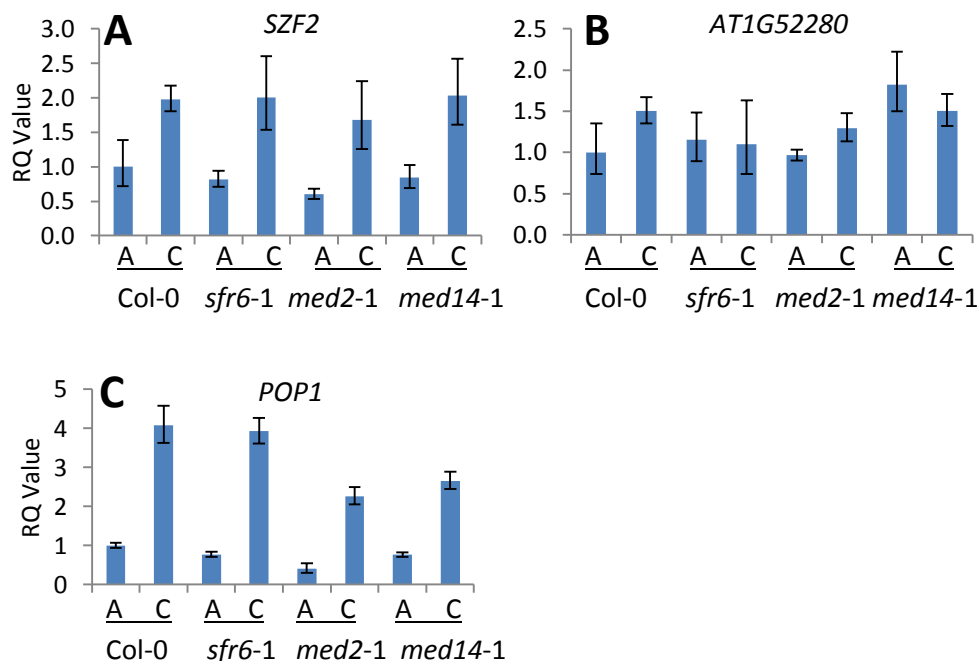


Figure 13: Relative quantification (RQ value) of cold-inducible transcript levels from genes that are non-misregulated in *sfr6* in response to cold. A: Ambient (20°C) C: Cold (5°C). Genes are induced in response to 6h at 5°C or 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

As indicated by the microarray, these genes were induced in response to cold and did not show an impaired cold-induced expression profile in *sfr6-1* seedlings despite the loss of the SFR6/MED16 Mediator subunit. *POP1* (At5g44110) shows slight impairment in expression in *med2-1* and *med14-1* lines, but not *sfr6-1* mutants (Figure III.13C).

III.5.1.5. *AT5G54470*-like genes

The cold-inducible *AT5G54470* gene (previously identified in a search for non-CBF regulated cold-inducible genes) has been shown to be misregulated in *sfr6* mutants in cold conditions but not *med2-1* or *med14-1* mutants (personal comm. P. Hemsley). As this gene is so far unique in showing misregulation only in *sfr6* mutants and not also in *med2-1* or *med14-1* mutants, other genes misregulated in *sfr6* to a similar extent as *AT5G54470* that contained the EE motif (AAAATATCA) on the microarray were investigated by qRT-PCR to see if they showed a similar gene expression profile to *AT5G54470* of impaired expression only in *sfr6* alleles and not in *med2-1* or *med14-1*.

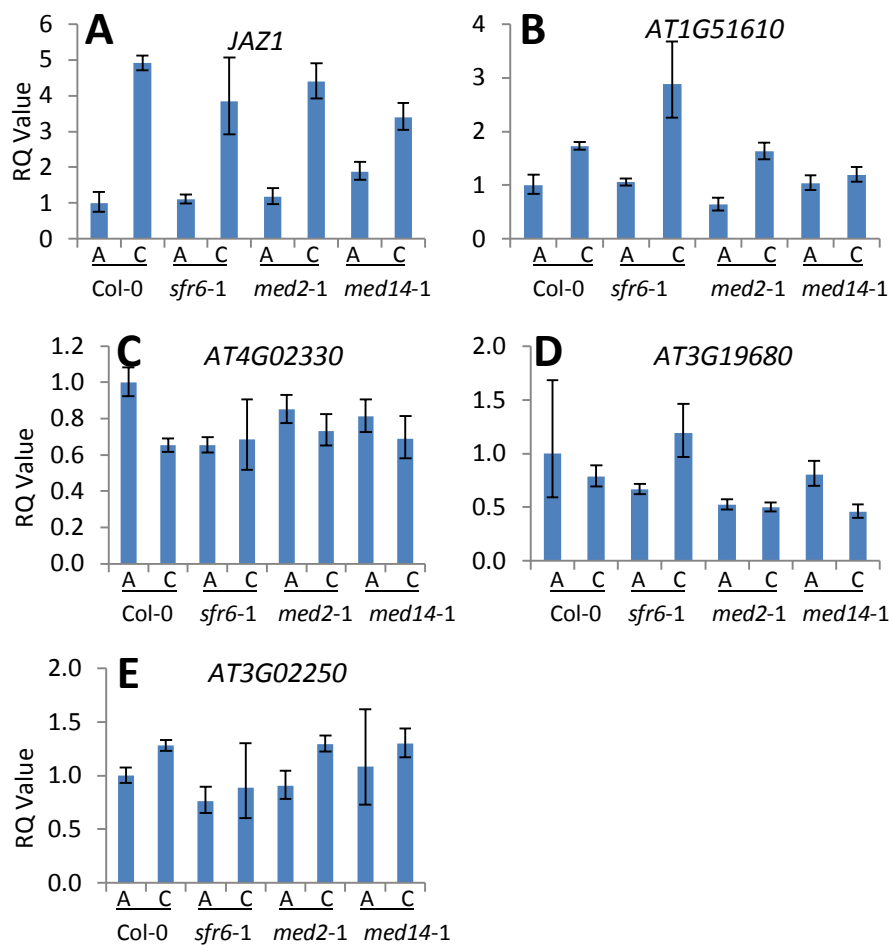


Figure 14: Relative quantification (RQ value) of cold-inducible transcript levels of *AT5G54470*-like genes under ambient and cold conditions. A: Ambient (20°C) C: cold (5°C) Genes are induced in response to 6h at 5°C or 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments

Data in Figure III.14 shows that the *AT5G54470*-like genes were not, in the main, cold-inducible in wild type seedlings and were not shown to be misregulated in *sfr6-1* as

suggested by the microarray. The pattern of gene expression was not consistent with the *AT5G54470* gene with a reduced expression in *sfr6-1*, but not in *med2-1* or *med14-1* lines.

III.5.2. Chromatin Immunoprecipitation (ChIP) Experiments

ChIP experiments were carried out to investigate Pol II recruitment to different sites along the length of cold-inducible genes in wild type, *sfr6-1*, *med2-1* and *med14-1* mutants under cold and ambient conditions to see if Pol II occupancy was compromised in the mutant lines, leading to the reduced transcript levels seen in CRT/DRE- and EE-containing genes.

III.5.2.1. ChIP Full Length

Chromatin immunoprecipitation (ChIP) experiments were carried out using an antibody against the c-terminal domain (CTD) repeats of RNA Polymerase II (Pol II) to investigate Pol II recruitment along the length of cold-inducible genes *KIN2* (CRT/DRE-containing gene) and *AT1G20030* (EE-containing gene). Pol II occupancy at cold-inducible genes was normalised to the total input fraction of chromatin that had not been immunoprecipitated with anti-Pol II CTD antibodies for each sample and to the Pol II occupancy of *PEX4*, a housekeeping gene that does not alter its expression in response to cold, in each sample.

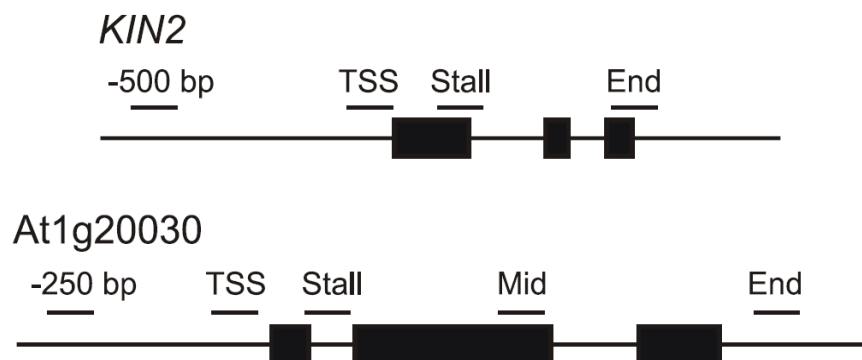


Figure 15: Primers used to investigate Pol II occupancy at 4 sites along the *KIN2* gene and 5 sites along the *At1g20030* gene. -500: 500 bp upstream of the predicted TSS; -250: 250 bp upstream of the predicted TSS; TSS: Transcriptional start site; Stall: predicted Pol II stall site; Mid: midpoint of the gene; End: End of the gene. Thick black bars represent exons, thin lines represent the region amplified by the primer pair.

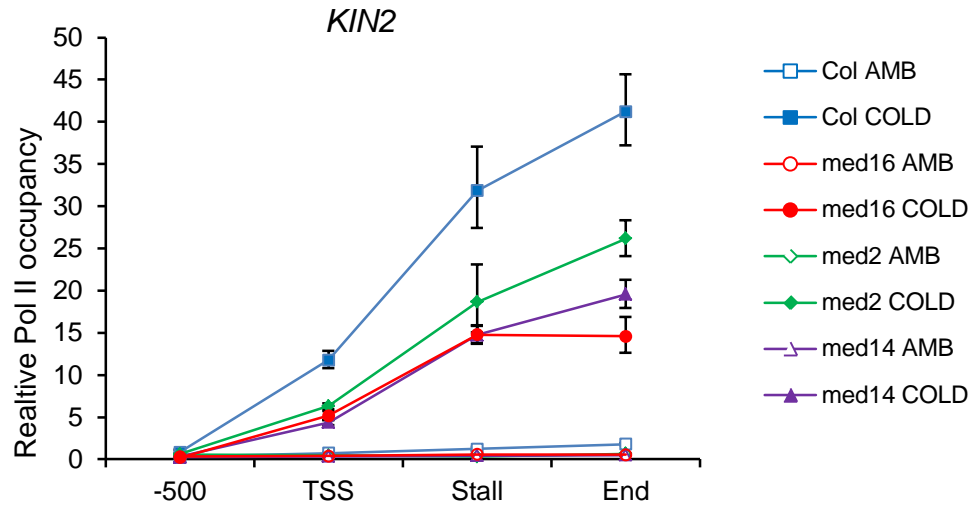


Figure 16: Relative occupancy of Pol II along the length of the *KIN2* gene at 4h under ambient (20°C) or cold (5°C) conditions. -500: 500bp upstream of the predicted transcriptional start site; TSS: predicted transcriptional start site; Stall: predicted stall site of Pol II; End: end of the gene. Cold: 4h at 5°C; Amb: 4h at 20°C. Error bars represent a 95% confidence interval of 3 technical repeats. Data is representative of two biological replicate experiments.

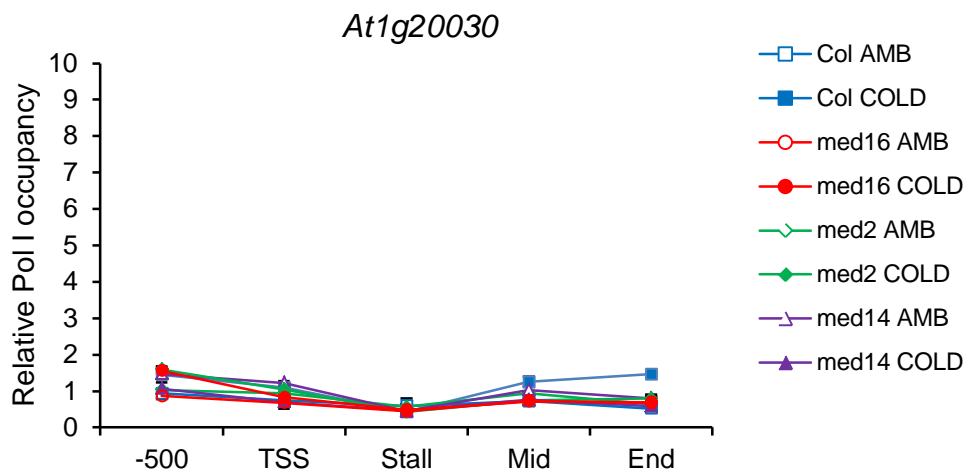


Figure 17: Relative occupancy of Pol I along the length of the *At1g20030* gene under ambient (20°C) and cold (5°C) conditions. -500: 500bp upstream of the predicted transcriptional start site; TSS: predicted transcriptional start site; Stall: predicted stall site of Pol II; Mid: midpoint of the coding sequence region; End: end of the gene. Cold: 4h at 5°C; Amb: 4h at 20°C. Error bars represent a 95% confidence interval of 3 technical repeats. Data is representative of two biological replicate experiments.

ChIP experiments to monitor the occupancy of Pol II along the length of *KIN2*, a CRT/DRE-containing gene showed that Pol II is recruited in response to cold (5°C) temperatures, but not in ambient (20°C) temperatures. Recruitment of Pol II was impaired in *sfr6-1*, *med2-1* and *med14-1* lines in the cold, compared to wild type. ChIP experiments along the length of *AT1G20030*, an EE-containing gene, showed that

recruitment of Pol II does not increase in response to cold and that there was no difference on Pol II occupancy between wild type seedlings and *sfr6-1*, *med2-1* or *med14-1* seedlings.

III.5.2.2. CRT/DRE genes

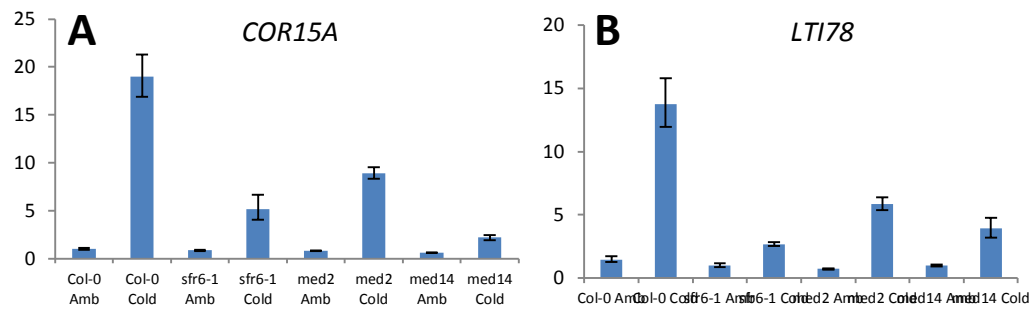


Figure 18: Relative occupancy of Pol II at the middle of the transcribed region of CRT/DRE-containing genes. Cold: 4h at 5°C; Amb: 4h at 20°C. ChIP was carried out against the CTD of Pol II at the midpoint of cold-inducible genes that contain a CRT/DRE element in their promoters but no EE motif. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

Further genes were examined at the midpoint position only chosen as full length ChIP experiments (Figure III.16 & 17) indicated that occupancy of Pol II was greatest at the midpoint of the transcribed region of the gene under cold conditions. Much like in *KIN2*, ChIP experiments on other CRT/DRE-containing genes, *COR15a* and *LTI78* (Figure III.18 A & B) revealed an increase in Pol II occupancy in the cold at the middle points of the transcribed region of cold-inducible genes in wild type plants that contain CRT/DRE promoter elements. Experiments showed reduced Pol II occupancy in *sfr6-1*, *med2-1* and *med14-1* mutant lines in the cold (5°C) compared to wild type.

III.5.2.3. EE only genes

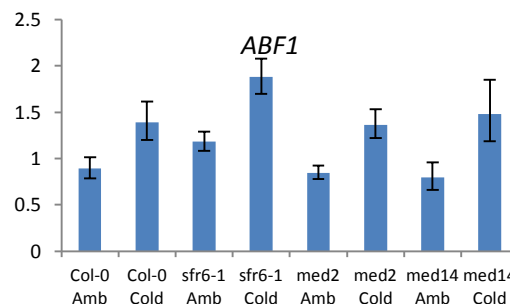


Figure 19: Relative occupancy of Pol II at the mid points of *ABF1* which contains an EE motif. Cold: 4h at 5°C; Amb: 4h at 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

III.5.2.4. Both CRT/DRE- & EE-containing genes

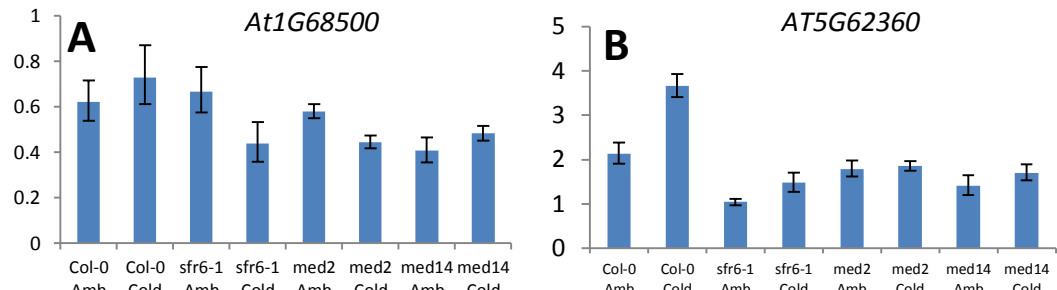


Figure 20: Relative occupancy of Pol II at the mid points of CRT/DRE- and EE-containing genes. Cold: 4h at 5°C; Amb: 4h at 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

Genes that contain the EE motif but no CRT/DRE promoter elements showed a less distinct pattern of Pol II recruitment in cold conditions at the midpoint of the gene compared to CRT/DRE genes (Figure 18). Presence of Pol II at midpoints in the cold was similar to that of ambient conditions in *ABF1* (Figure III.19). *AT1G68500* and *AT5G62360* are genes that contain both a CRT/DRE and EE element, *AT5G62360* showed a more CRT/DRE-like Pol II occupancy (Figure III.20B) but *AT1G68500* showed Pol II occupancy similar to EE genes (Figure III.20A). A microarray carried out by the Thomashow group (Fowler et al., 2002) indicated that *AT1G68500* and *AT5G62360* are not strongly upregulated by CBF2 overexpression despite the CRT/DRE promoter motif found present in this analysis. Therefore, they may not be genuine targets of the three CBF transcription factors and the CRT/DRE motifs found in their promoters may not constitute functional CBF-binding sites. This suggestion is supported by the observation that they are only mildly upregulated in the cold compared to other CBF-regulated genes such as *GOLS3* or *LT178*.

III.5.2.5. Non mis-regulated genes

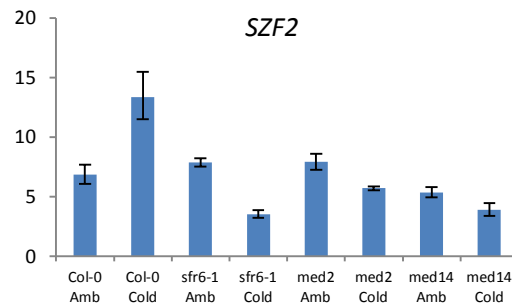


Figure 21: Relative occupancy of Pol II at the midpoint of *SZF2*, a gene not misregulated in response to cold in *sfr6-1*. Cold: 4h at 5°C; Amb: 4h at 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of one biological replicate experiment.

ChIP against the CTD of Pol II in *SZF2*, a gene not misregulated in *sfr6* in the cold (Figure III.21) compared to wild type showed an increase in Pol II occupancy in the cold (5°C) compared to the ambient (20°C). Surprisingly, experiments revealed reduced Pol II occupancy in *sfr6-1*, *med2-1* and *med14-1* lines in response to cold compared to the wild type.

III.5.3. *AT1G20030* alternative splicing

As Pol II recruitment to EE-containing genes was unimpaired in *sfr6-1*, *med2-1* and *med14-1* compared to wild type, posttranscriptional modifications to the *AT1G20030* transcript were investigated in wild type and the three mutant lines with a view to uncovering the reason for the differential transcript levels observed in the cold. In response to abiotic stress, alternative splicing of mRNA transcripts can occur to allow expression and translation of required stress-responsive genes (Chinnusamy *et al.*, 2007). *AT1G20030* is a cold-inducible gene (Figure III.8E) that has two predicted protein-coding gene models and multiple cDNA variants (Figure III.22 A & B). Therefore, a proposed mechanism for impaired expression of *AT1G20030* in *sfr6-1*, *med2-1* and *med14-1* seen in cold conditions (Figure III.10A) was that the increased transcript levels seen in the cold was an artefact of primers detecting a different splice variant in response to cold rather than a true increase in transcript levels in response to cold.

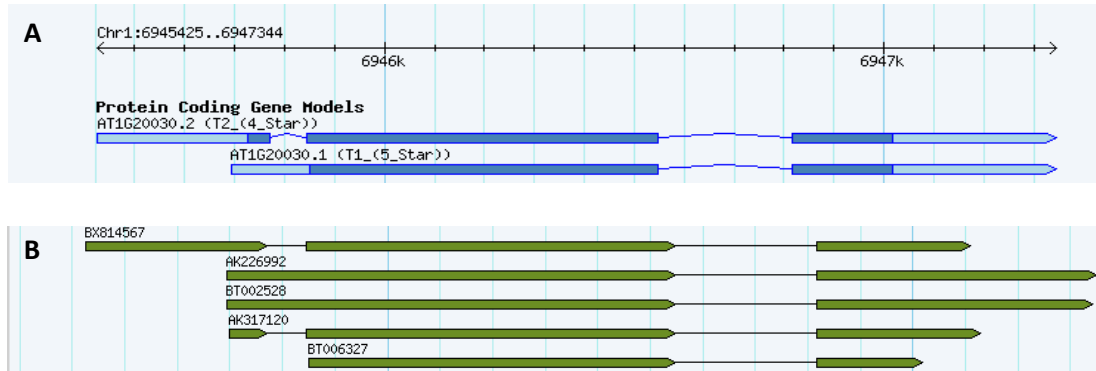


Figure 22: Two protein coding gene models of *At1G20030* (A). Different cDNA variants of the *At1G20030* gene (B). Data from <http://www.arabidopsis.org>.

Specific primers were therefore designed to detect three different splice variants of the *AT1G20030* gene to investigate alternative splicing events in *sfr6-1*, *med2-1* and *med14-1* mutants in response to the 6h of cold (5°C) or ambient (20°C) conditions used in previous qRT-PCR experiments (Figure III.23).

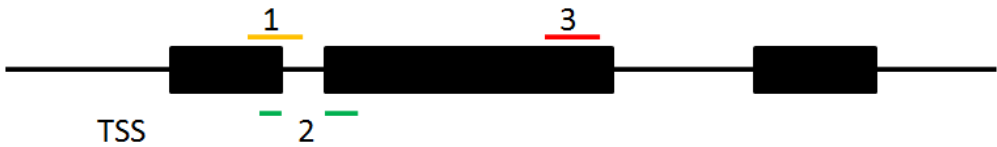


Figure 23: Gene map depicting primers to detect different splice variants of *AT1G20030*. TSS: transcriptional start site; 1: primers span the start site of the gene and are used in qRT-PCR experiments; 2: primers span the first small intron; 3: primers an exon region and are used in ChIP experiments as the midpoint of *AT1G20030*. Thick black lines represent exons, thin coloured lines represent the region of PCR product amplified by the primer pair

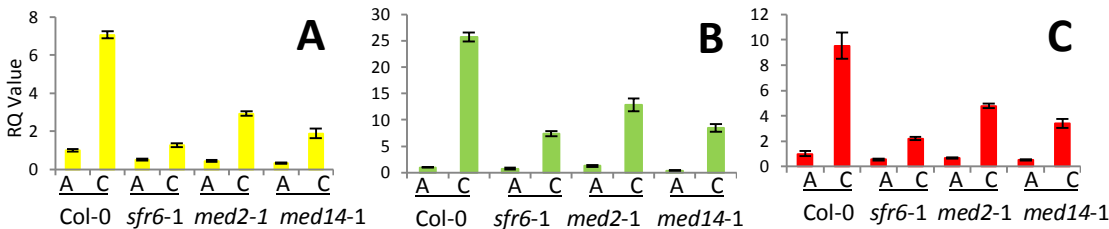


Figure 24: Relative quantification (RQ value) of different splice variants of the *At1g20030* gene under ambient and cold conditions. Colours represent the 3 different primer pairs shown in Figure 23. A: Ambient (20°C) C: cold (5°C). A: Primer set 1 used in qRT-PCR experiments; B: Primer set 2 that span the small intron; C: Primer set 3 used in ChIP experiments. *AT1G20030* is expressed in response to 6h at 5°C or 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments. A) Thom10 spliced B) Thom10 real-time primers C) Thom10 ChIP primers

From these data, it appeared that relative levels of the different splice variants did not change in the cold and cannot therefore account for the increase in one type of transcript in the cold (Figure III.10A). These data also showed that alternative splicing events are occurring in the *AT1G20030* gene, but that splicing is not impaired in *sfr6-1*, *med2-1* or *med14-1* mutants in response to cold treatment (Figure III.24 A-C).

III.5.4. CRT::LUC transformations

Experiments have previously shown that cold-inducible expression via the CRT/DRE motif is impaired in *sfr6* mutants (Boyce *et al.*, 2003). Boyce *et al.* conducted experiments using a concatamer reporter construct in which 4 copies of the motif were fused to an unmodified luciferase sequence that resulted in the emission of relatively low levels of light that are not easily detected. Whalley *et al.* (2011) later produced a version of this construct in which the modified codon optimised LUC+ version of luciferase was used. This gives greater levels of luciferase protein expression that were high enough for transient expression experiments to be attempted as expression of reporter genes is greatly reduced in a transient system than in stable transformant lines. The LUC+ construct was used to attempt transient expression experiments using *med2-1* and *med14-1* mutants. The artificial concatamer contains a -70 minimal promoter and 4 copies of the CRT/DRE motif fused to a *LUCIFERASE* reporter gene (Figure II.1). *med2-1* and *med14-1* lines were transiently transformed with the CRT::LUC+ concatamer and tested for reporter gene expression in response to cold treatment (24h at 5°C) prior to imaging to see if the MED2 and MED14 subunits were required specifically for activation by cold via the CRT promoter. Luciferase activity in the form of photons released is measured as a proxy for CRT-driven expression levels. The level of activity is reflected in the numbers of photons captured (raw photon counts).

III.5.4.1. CRT::LUC bombardment into wild type seedlings

In a proof-of-concept experiment, 7 day old wild type seedlings were transiently transformed by biolistics to express a CRT::LUC+ concatamer (Whalley *et al.*, 2011). Seedlings were treated with either cold (5°C) or ambient (20°C) temperatures for 24h,

returned to ambient conditions for 1h and then the luciferase luminescence was imaged for 10 minutes to reveal differences in expression levels.

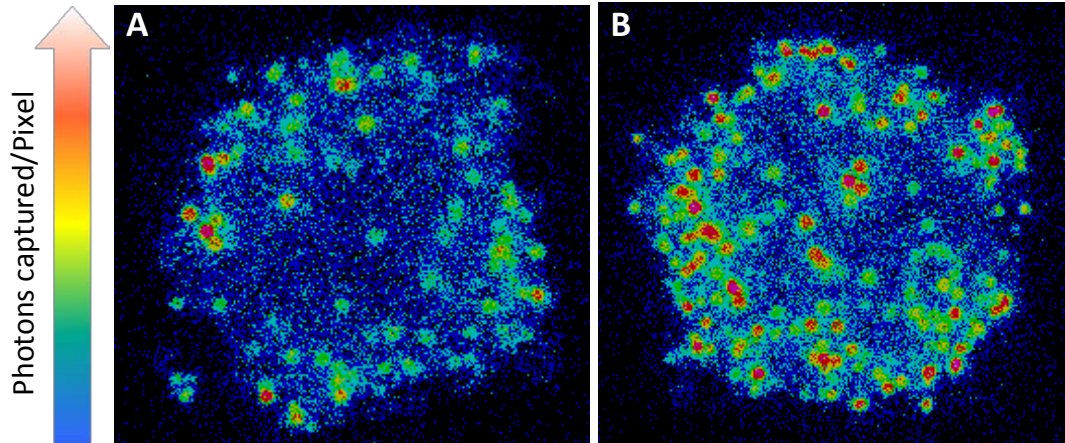


Figure 25: Photons captured during 10 minutes of imaging from luciferase luminescence in 7 day old seedlings expressing a CRT::LUC+ concatamer in ambient or cold conditions. Cold: 24h at 5°C (B); ambient: 24h at 20°C (A). Seedlings were sprayed with 5mM luciferin in 0.01% Triton-X and imaged for 10 minutes. Image is in false colour and representative of 3 biological replicate experiments.

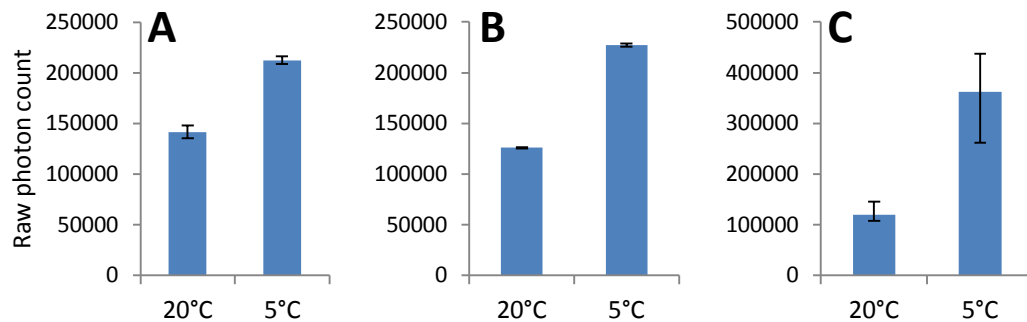


Figure 26: Average photon counts from CRT::LUC+ bombardment into wild type seedlings. Error bars represent the range of photon counts collected from the 2 technical repeats of each bombardment over a period of 10 minutes. Data shown is from each of the three biological replicate experiments.

This proof-of-concept experiment showed that more photons were captured from seedlings that had been treated with 5°C (Figure III.25B) for 24h than in the control conditions of 20°C (Figure III.25A) for 24h. Raw photon count data (Figure III.26B) is captured from luciferase luminescence images (Figure III.25) taken during imaging and shows an identical pattern of fewer photons captured in seedlings under ambient conditions than cold conditions.

III.5.4.2. CRT::LUC bombardment into 3 mutant lines

Having shown that transiently expressed CRT::LUC+ concatamer construct showed a greater degree of expression in the cold than in ambient conditions in wild type plants (Figure III.25 & 26), expression of the concatamer was investigated in *sfr6-1*, *med2-1* and *med14-1* seedlings in cold and ambient conditions.

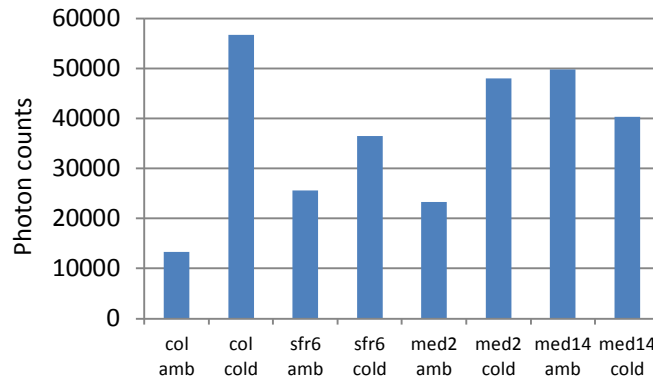


Figure 27: Number of photons captured from bombarding a CRT::LUC concatamer construct into wt, *sfr6*, *med2-1* and *med14-1* mutant lines. Cold: 24h at 5°C; Amb: 24h at 20°C. Data is representative of a single biological replicate.

In the first experiment, the wild type, *sfr6-1* and *med2-1* all show a similar of increased numbers of photons captured in the cold compared to the ambient. Preliminary data suggest that the *med2-1* and *sfr6-1* lines show reduced numbers of photons captured in the cold compared to the wild type. The *med14-1* line showed a reduced photon count in the cold compared to ambient, however the numbers of photons captured in both the ambient and cold samples were strangely more elevated than either the *med2-1* or *sfr6-1* lines (Figure III.27). This was likely due to differences between coating the DNA onto the gold particles for delivery and the amount of DNA-coated gold particles bombarded into each seedling set. A control for transformation efficiency between samples was not possible, however, further experiments described below attempted to address this problem.

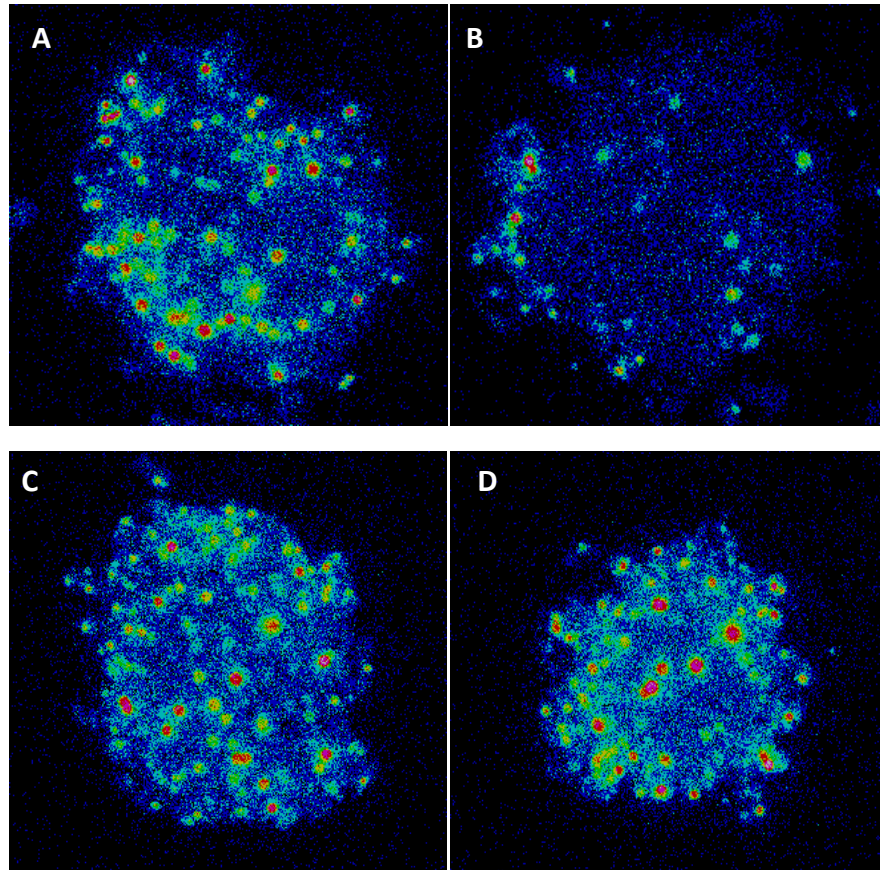


Figure 28: Photons captured from wild type (A), *sfr6-1* (B), *med2-1* (C) and *med14-1* (D) seedlings bombarded with a CRT/DRE::LUC concatemer construct after 24h in the cold. Data is representative of two biological replicate experiments and two technical replicates in each biological replicate.

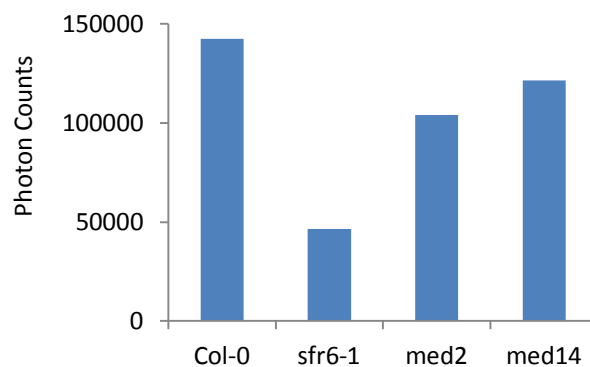


Figure 29: Photon counts from a second biological replicate experiment of CRT::LUC bombarded into wild type, *sfr6-1*, *med2-1* and *med14-1*. Seedlings were treated with 24h cold (5°C) prior to imaging. Data is representative of a single biological replicate experiment shown in Figure III.28.

In a second experiment, only cold-treated plants were compared between the 4 plant lines. Data from photon counts captured from expression of the LUC+ reporter gene (Figure III.28) showed a pattern of fewer photons captured in *sfr6-1*, *med2-1* and

med14-1 in the cold than in wild type seedlings in the cold in both experiments (Figure III.29). This suggests that *sfr6-1*, *med2-1* and *med14-1* have an impaired ability to activate expression of LUC+ via the CRT/DRE promoter element compared to wild type seedlings.

III.5.4.2.1. Analysis of CRT::LUC transcript levels in WT, *sfr6-1*, *med2-1* and *med14-1* by qRT-PCR

As there was no way to control for the number of cells transformed by each bombardment, photon count data is not a reliable method for quantifying relative expression via the CRT in different plant lines. Therefore, LUC+ transcripts were further investigated to elucidate whether the luciferase activity previously measured (Figure III.29) was due to the differences in number of transformed cells or due to increased expression of the concatemer in the same number of cells. It is usually the case that LUC+ emission acts as a good proxy for transcript abundance (Millar *et al.*, 1992) and relative transcript levels of LUC+ was therefore investigated by qRT-PCR analysis to confirm this. An *AEQUORIN* gene under the control of a constitutive 35S:: promoter (Knight *et al.*, 1991) was transiently co-expressed with LUC+ as a control for the varying levels of transformation efficiency between samples. qRT-PCR experiments were carried out on LUC+ transcripts in wild type, *sfr6-1*, *med2-1* and *med14-1* seedlings under cold and ambient conditions and data was normalised against the expression of co-expressed *AEQUORIN*.

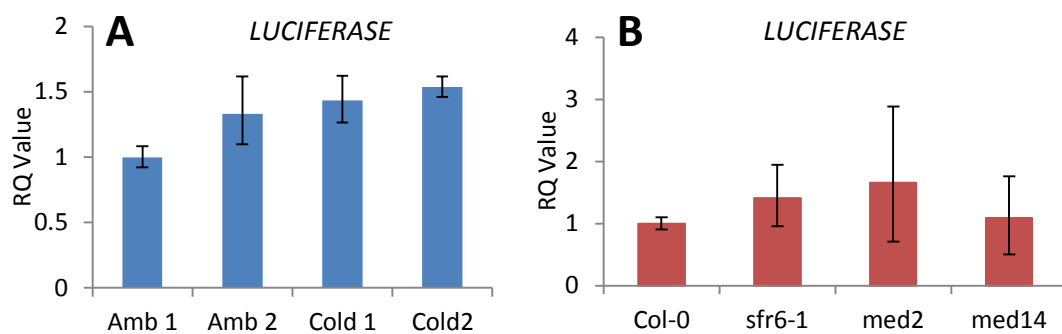


Figure 30: A: relative quantitation (RQ value) Luciferase transcript levels in wild type seedlings in response to 24h of either 5°C or 20°C. A: Ambient (20°C); C: Cold (5°C). **B:** Relative quantitation (RQ value) of LUC+ transcripts in wild type, *sfr6-1*, *med2-1* and *med14-1* seedlings after 24h at 5°C. Expression of LUC+ is normalised to expression of aequorin driven by the 35S:: promoter.

Despite the differences seen in luminescence captured from wild type seedlings (Figure III.26 A-C) in either cold (5°C) or ambient (20°C) conditions, LUC+ transcript levels, when normalised to aequorin expression, were not shown to be elevated in the wild type in cold, compared to ambient samples. Similarly, expression of LUC+ is not reduced in *sfr6-1*, *med2-1* or *med14-1* seedlings in the cold compared to wild type (Figure III.30B).

III.5.4.3. Protoplasts

Protoplast isolation and transient transformation with the CRT::LUC+ concatemer was attempted as a more reliable system to empirically test transcription of LUC+ in wild type, *sfr6-1*, *med2-1* and *med14-1* Arabidopsis. The transformation required protoplasts at a concentration of 5×10^6 in 300µl, however in both attempts at protoplast extraction, insufficient protoplasts were recovered to transform. Measurements using a haemocytometer showed that a total of fewer than 1×10^6 protoplasts were recovered after the final centrifugation step.

It was found that many intact protoplasts were lost in the decanted supernatant after each wash, while protoplast debris was also found in the supernatant. This suggests that after each resuspension, protoplasts could be centrifuged for longer at the same gravity to collect more of the protoplasts. To avoid excessive loss of protoplasts, the supernatant could also be removed from the falcon tubes without removing them from the centrifuge to avoid accidental resuspension of protoplasts while transporting them. Due to time constraints, it was not possible to attempt further protoplast isolation and transient transformation experiments with the suggested optimisations.

III.6. Expression of sugar-inducible cold-responsive genes

Using the RSAT analysis software to investigate promoter elements in the group of 81 misregulated genes, the AAATATC promoter motif was found. Literature suggests that it is an EVENING ELEMENT-like sequence (AAAATATCA) present in promoters of sugar-responsive genes (Tatematsu et al., 2005) and was thus called the SUGAR RESPONSE ELEMENT (SRE). Preliminary experiments were first carried out on wild-type *Arabidopsis thaliana* plant lines, looking at expression of known cold-inducible genes

from the microarray and qRT-PCR analysis that also contained the AAATATC motif, in response to the presence of sucrose in light or dark conditions.

Unless otherwise stated, all gene expression is normalised to the expression of *PEX4*, a housekeeping gene that does not alter its expression in response to most abiotic stresses (Czechowski *et al.*, 2005). Graphs show relative quantitation (RQ value) of gene expression compared to the first temperature-treated sample on the histogram (set to a value of 1), as described by the $\Delta\Delta C^T$ method (Livak & Schmittgen 2001) of transcript quantitation. Patterns of gene induction in response to sugar were consistent across two biological replicate experiments, however slight variation was observed in the degree of gene induction between experiments. Unless otherwise stated, a representative experiment for each gene has been shown. Purple bars indicate the sucrose treatment (3% w/v) while blue bars indicate the mannitol control (0.096M, an equisomolar concentration of a non-metabolisable sugar) represent the dark-treated samples, light blue and purple represent light-treated samples. Darker blue and purple represent the dark-treated samples, light blue and purple represent light-treated samples.

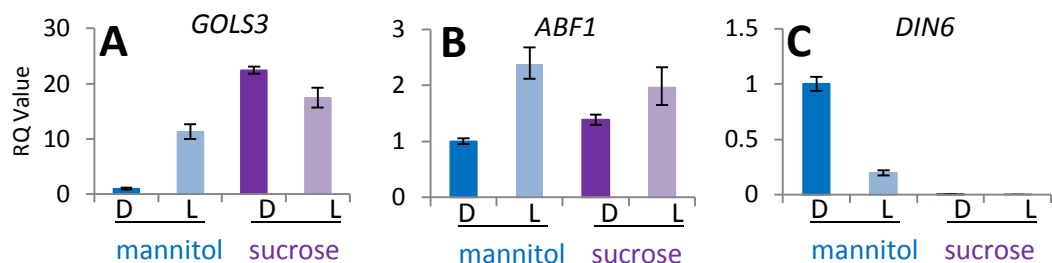


Figure 31: Relative quantification (RQ value) of transcripts that contain the AAATATC promoter motif in 7-day old seedlings treated with isoosmotic concentrations of mannitol or sucrose in the light or dark for 6h. L: Light, D: Dark. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

DIN6 is a control gene that is known to be induced in the dark (Buchanan-Wollaston *et al.*, 2005), and its induction in the dark was repressed by sugar in wild type seedlings, as shown in Figure III.31C, showing each treatment was correctly experienced by the seedlings.

Data from the wild type seedlings suggested that genes with an EVENING ELEMENT (EE: AAAATATCA) promoter motif were more upregulated in response to light than

sucrose (Figure III.31B). Genes with the SRE (AAATATC) and C-REPEAT/DROUGHT RESPONSE ELEMENT (CRT/DRE: CACGTG) promoter motif were more responsive to the presence of sugar than light as genes are equally expressed in the dark in the presence of sucrose, as in the presence of mannitol in the light (Figure III.31A).

Further experiments were then carried out on *sfr6-1*, *med2-1* and *med14-1* seedlings, to compare their gene expression in response to sucrose in dark or light conditions with that of wild type. The wild type samples in these experiments were those described above (Figure III.31)

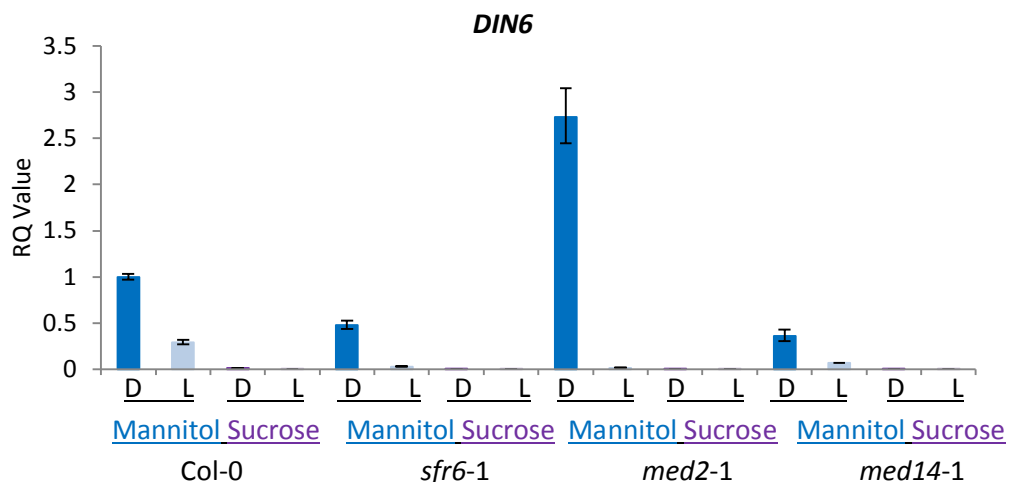


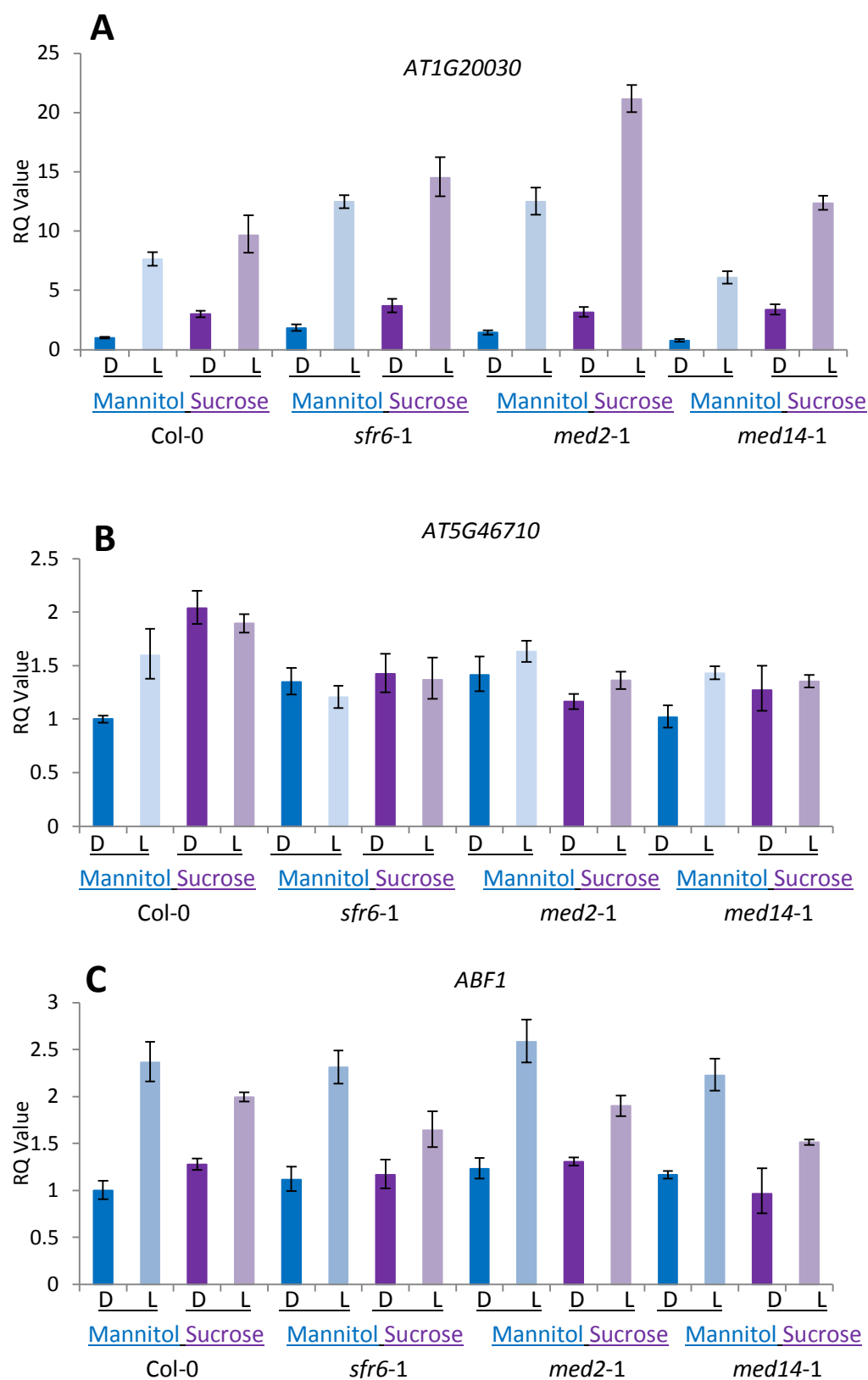
Figure 32: Relative quantification (RQ value) of a known dark-inducible gene in response to sugar in the light or dark. D: dark; L: light. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of two biological replicate experiments.

DIN6 was used as a control gene known to be induced in response to dark and repressed in the presence of sugar or light, the gene expression profile seen in the wild type of this experiment, indicating that treatments were successfully experienced by the seedlings (Figure III.32). Expression of *DIN6* in *med2-1* was elevated under dark and mannitol treatments compared to wild type, *sfr6-1* and *med14-1* samples, suggesting that the MED2 subunit is not required for sugar-induced expression of *DIN6*.

III.6.1. EE genes

Relative expression of genes that were previously shown to have an EE promoter motif in addition to the SRE (AAATATC) motif was investigated in wild type, *sfr6-1*, *med2-1*

and *med14-1* seedlings in response to the presence of sucrose in the light or dark (Figure III.33).



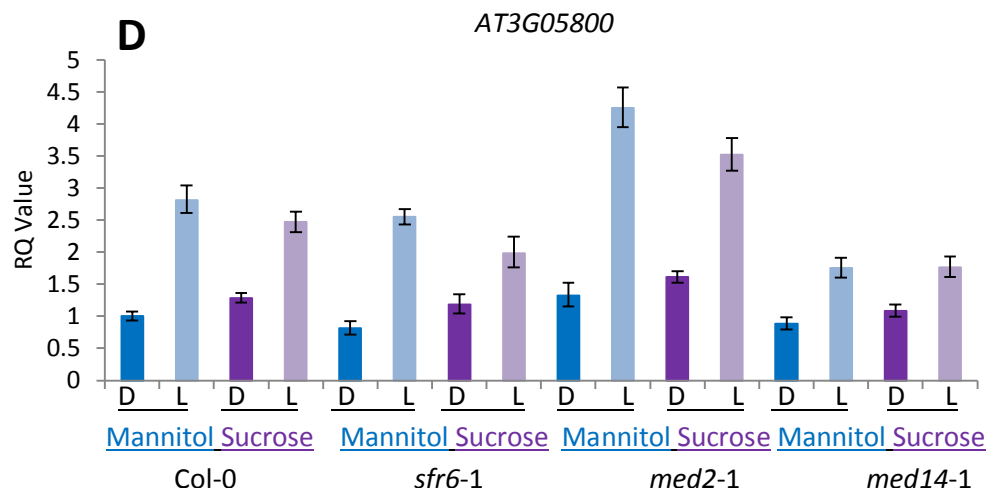


Figure 33: Relative quantitation (RQ value) of EE-containing genes in response to sucrose in the light or dark for 6h. D: Dark; L: Light. Error bars represent a 95% confidence interval of 3 technical repeats. Data is representative of two biological replicate experiments.

III.6.2. CRT/DRE genes

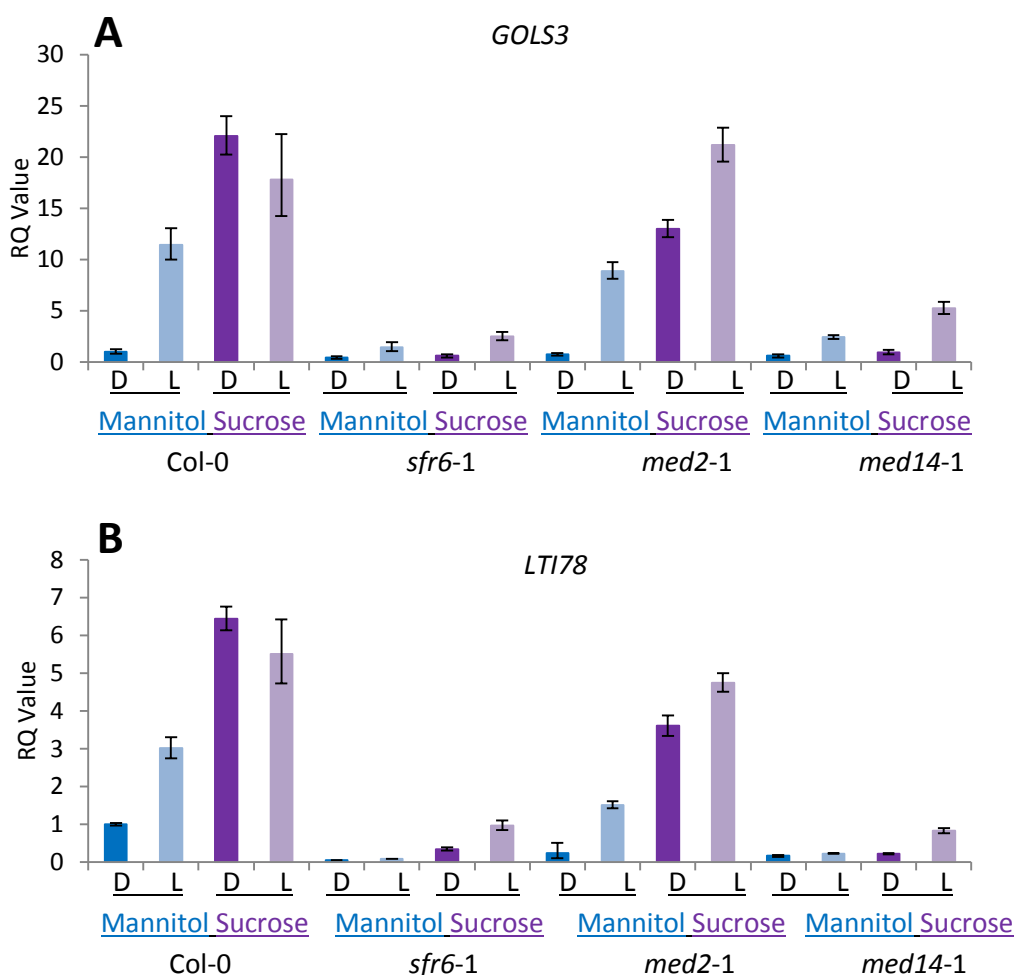


Figure 34: Relative quantification (RQ value) of CRT/DRE-containing genes in response to sugar or mannitol in the light or dark. D: Dark; L: Light. Error bars represent a 95% confidence interval. Data is representative of two biological replicate experiments.

Data from this experiment show that genes with an EE were not strongly misregulated in any of the mutant lines in the response to sugar (Figure III.33A-D). EE-containing genes (*AT1G20030*, *AT5G46710*, *ABF1* & *AT3G05800*) were induced to a greater degree in response to light compared to sugar (Figure III.33). Genes with a CRT/DRE motif (*GOLS3*, *LT178*) were misregulated in *sfr6* and *med14-1* lines, but not in *med2-1* (Figure III.34A & B). cold-responsive CRT/DRE-containing genes were more strongly induced in response to sucrose rather than light. Sugar responses in genes that contain a CRT/DRE promoter motif have not previously been shown with exception to *GOLS3*. This experiment showed that the SFR6 and MED14 subunits are required for gene expression in response to light and sugar, but that the MED2 subunit was not required (Figure III.33 & 34). This suggests that different sets of subunits are required for the transcriptional response to different abiotic stresses.

III.7. Double mutant lines: *sfr6-1*, *med2-1* and *med14-1*

Having shown that expression of cold-inducible genes in *sfr6-1*, *med2-1* and *med14-1* lines is impaired in response to cold, light and sugar, crosses were made between the three mutant lines to create plant lines with pairs of the three subunits knocked out. This was to investigate whether SRF6/MED16, MED2 and MED14 Mediator subunits have overlapping synergistic or redundant functions in response to abiotic stresses or if each subunit has a unique role in the response to abiotic stress.

III.7.1. Genotyping for *sfr6-1*, *med2-1* and *med14-1* double mutants

III.7.2 Genotyping and Mendelian genetics

According to Mendelian genetics as *SFR6/MED16*, *MED2* and *MED14* are on different chromosomes, it would be expected that 1/16 of all F₂ generation plants from each cross tested would be homozygous mutants for both Mediator subunit genes, assuming an equal inheritance of both genes. Two separate reciprocal crosses were made of each mutant pair (data not shown).

III.7.2.1 *med2-1* x *med14-1*

26 plants from the first *med2-1* x *med14-1* cross were genotyped by PCR using gene-specific primers. 14 plants were found to be *med2-1* homozygous, but none of which were found to be *med2-1med14-1* double mutant lines.

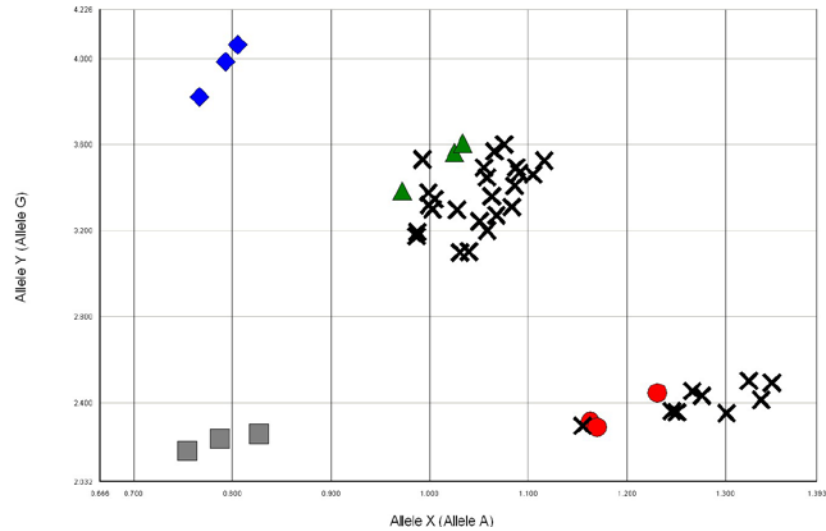
III.7.2.2. Genotyping for the *sfr6-1* EMS point mutation

Figure 35: *sfr6-1* genotyping of homozygous *med2-1* and *med14-1* mutant lines. ▲ represents known heterozygous *sfr6* lines (1 copy of the mutant *sfr6* allele) , ◆ represents known *sfr6-1* homozygous EMS mutant lines (2 copies of the mutant *sfr6* allele), ● represents known wild-type lines (2 copies of the wild type *SFR6* allele). X represents plant lines of an unknown *sfr6* mutant status to be genotyped. ■ represents no template controls. The X and Y axes show the relative frequency of the different allele forms. Three technical repeats were carried out for each plant line. Each symbol is representative of three technical replicate reactions.

Genotyping for the *sfr6-1* mutation was carried out using a Taqman® genotyping probe that can detect single base pair changes present in the genomic DNA of *sfr6-1* EMS mutants. *sfr6-1* mutants contain a point mutation in the *SFR6* genes that causes a premature stop codon early in the coding sequence of the protein. The x-axis shows the relative number of mutant *sfr6* allele copies in the genomic DNA; the y-axis shows the relative number of wild type *SFR6* allele copies present. Putative *sfr6-1* plants lines (X on Figure III.35) are compared with known *sfr6-1* homozygote (◆), *sfr6-1* heterozygote (▲) or wild type lines (●), indicating how many copies of the *SFR6* allele they carry. The proximity of an unknown sample plant (X) to the known *sfr6-1* (◆) indicates whether the plant is an *SFR6*, *sfr6-1* or a heterozygote.

III.7.2.2.1. *med2-1 x sfr6-1*

51 *med2-1 x sfr6-1* plants were selected for genotyping based on an unbiased selection. While 20 were found to be *med2-1* homozygous mutants, none of these plants were subsequently found to also be homozygous for the *sfr6-1* mutation.

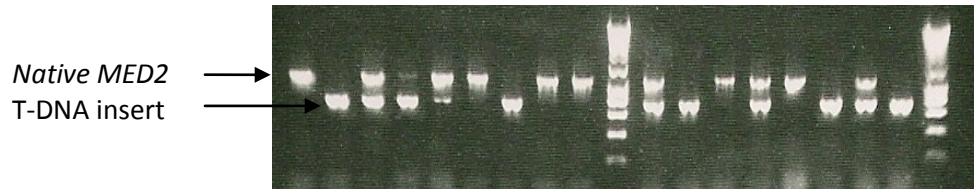


Figure 36: Electrophoresis gel showing the PCR product of the native *MED2* gene Forward/Reverse primer pair (upper bands) and the T-DNA insert SAIL LB/Reverse (lower bands) primer pair used to identify *med2-1* homozygotes.

Seeds from the reciprocal *sfr6-1 x med2-1* cross were then grown, from which 17 plants were selected for the *sfr6-1* visible phenotype (larger, yellow cotyledons) (Knight et al, 2009) and screened for the T-DNA insert in *MED2*.

Elimination of the seedlings with a wild type phenotype and specific selection of the *sfr6-1* phenotype (Knight *et al.*, 2009) increased the chances of finding a double mutant plant as the selected seedling was already likely to be *sfr6-1* mutant. One plant line was found to be a *med2-1sfr6-1* double mutant. This plant had a severely dwarfed phenotype and was extremely slow growing compared to its wild-type and single mutation counterparts (Figure III.38).

III.7.2.2.2. *med14-1 x sfr6-1*

Similarly to the *med2-1 x sfr6-1* double mutant screening, 51 *med14-1 x sfr6-1* plants were selected on an unbiased basis and genotyped by PCR for the T-DNA insert in *MED14*. Eight of these plants were found to be *med14-1* mutants, none of which were subsequently found to be homozygous for the *sfr6-1* mutation.

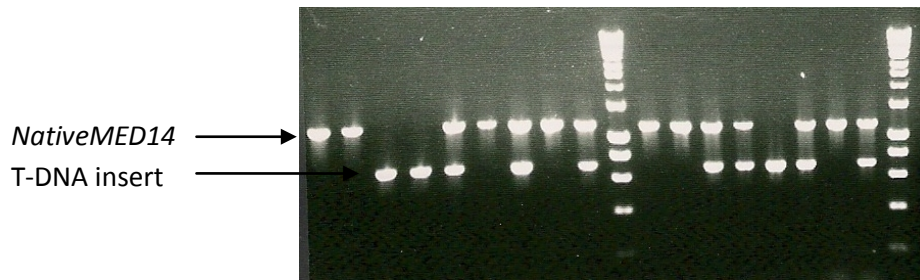


Figure 37: electrophoresis gel showing the PCR product of the native *MED14* gene forward/reverse primer pair (upper bands) and T-DNA insert SALK LB/ reverse (lower bands) primer pair used to identify *med14-1* homozygous lines.

As in the reciprocal *sfr6-1* x *med2-1* cross, seeds from the reciprocal *sfr6-1* x *med14-1* cross were grown and 17 plants were selected based on the *sfr6-1* visible phenotype and screened for the *MED14* T-DNA insert by PCR. Only one plant was found to be homozygous for both the *med14-1* T-DNA insert and the *sfr6-1* EMS mutation.



Figure 38: *sfr6* (left), *med14-1sfr6-1* (middle) and *med2-1sfr6-1* (right) mutant lines at 5 weeks old.

The *med14-1sfr6-1* double mutant plant showed the visible *sfr6-1* phenotype (Knight *et al.*, 2009) and in additions it was slightly dwarfed compared to *sfr6-1* plants (Figure III.38). The *med2-1sfr6-1* double knock out plant showed a severely dwarfed phenotype at 5 weeks old, compared to *sfr6-1* and the *med14-1sfr6-1* double knock out line. It was therefore not possible to carry out any freezing tolerance or gene expression experiments on the *med2-1sfr6-1* or *med14-1sfr6-1* double mutant lines in the time available.

III.8. Other Mediator subunit mutants

III.8.1. NRB4/MED15

NRB4/MED15 (Canet *et al.*, 2012) is another predicted Mediator tail subunit that has been shown to interact with transcription factors in yeast. Studies show that the yeast MED15 subunit can bind the yeast MED2 Mediator subunit (Mathur *et al.*, 2011). Due to the similarities between the plant and yeast Mediator complex, it is possible that the plant NRB4/MED15 and plant MED2 proteins also interact in the plant Mediator complex.

Two confirmed *nrb4/med15* T-DNA insert lines are also available from NASC but have been shown to be sterile and are seedling lethal in the absence of sucrose so cannot be grown on soil for propagation: in each generation, the parent must be heterozygous for the mutation and the homozygous mutants selected for as seedlings (Canet *et al.*, 2012). Therefore, these seed lines were not used in any of the experiments presented here as many seedlings are required for gene expression analysis when the seedlings are 7-days old and individual homozygous seedlings could not be identified and harvested rapidly enough for experimental purposes. In addition, the risk of failing to eliminate all wild-type seedlings from the experiment was too great and could have created artefacts in the data. *nrb4* mutants were not included in the original cold-response experiments as the seeds were a gift from the Tornero group in Valencia that arrived later in the year. The three *nrb4* mutant lines used are EMS mutants that contain three different SNPs which cause amino acid alterations in the KIX domain of the NRB4 protein (Figure II.4). These three *nrb4* EMS mutant lines were previously shown to have impaired responses to pathogenesis (Canet *et al.*, 2012).

III.8.1.1. Quantitative real time PCR- Cold

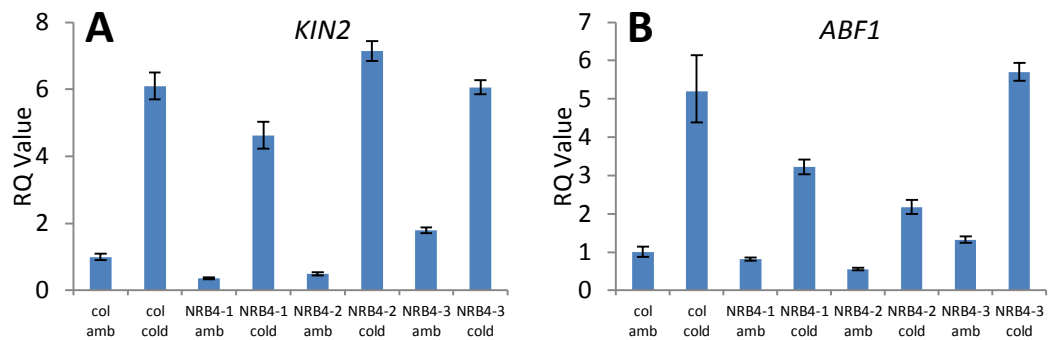


Figure 39: Relative quantification (RQ value) of cold-inducible transcript levels in 3 separate *nrb4/med15* lines in cold or ambient conditions. Cold: 6h at 5°C; Amb: 6h at 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is representative of one biological replicate experiment.

Preliminary experiments were carried out on all three *nrb4/med15* EMS mutant lines under cold (5°C) and ambient (20°C) conditions. The *nrb4-1* allele showed impaired expression of the cold-inducible genes *KIN2* and *ABF1* (Figure III.39). The *nrb4-2* allele showed different impairments in expressing *KIN2* and *ABF1*. The *nrb4-3* line did not appear to show any impairment in cold-induced gene expression.

Further experiments testing the role of NRB4/MED15 in the responses to other abiotic stresses were carried out only on the *nrb4-1* and *nrb4-3* alleles as too few *nrb4-2* seeds were available for experiments.

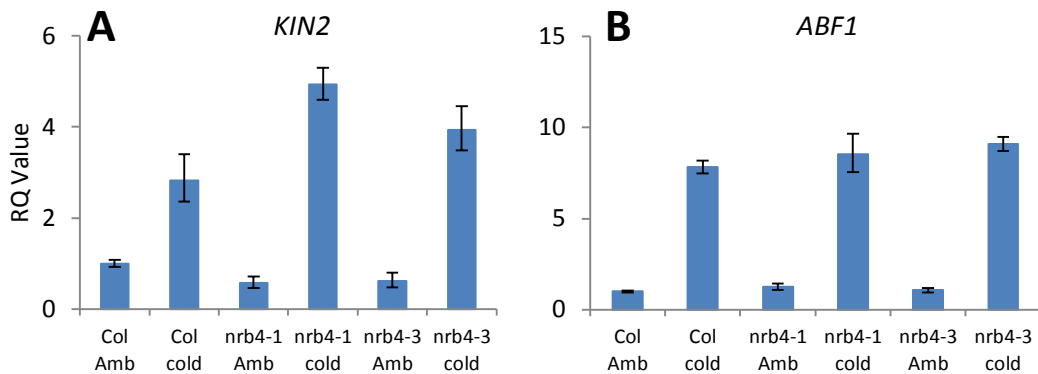


Figure 40: Relative quantification (RQ value) of cold-inducible transcripts in cold or ambient conditions. Cold: 6h at 5°C; Amb: 6h at 20°C. Error bars represent a 95% confidence interval from 3 technical replicates. Data is representative of two biological replicate experiments.

Data from two further biological repeats suggested that the preliminary experiments showing *nrb4/med15* lines were impaired in expressing cold-inducible genes was not

reproducible and therefore likely to be inaccurate (Figure III.40). Further experiments showed that cold-inducible genes are not down-regulated in either of the two *nrb4* lines (*nrb4-1* and *nrb4-3*) that were experimented with (Figure III.40).

III.8.1.2. Quantitative real time PCR– Dark & UV-C

Further experiments were carried out on *nrb4-1* and *nrb4-3* lines to investigate dark- and UV-C-induced transcripts. Dark-induced gene expression was normalised to the expression of *PEX4* (Peroxisomal ubiquitin conjugating enzyme 4), a housekeeping gene that does not alter its expression in response to many abiotic stresses (Czechowski *et al.*, 2005). UV-induced gene expression was normalised to the expression of *PR1* (At4g26410), a housekeeping gene that does not alter expression in response to UV-C as *PEX4* expression level have been shown to alter in response to UV-C (Wathugala *et al.*, 2012). Graphs show relative quantitation (RQ value) of gene expression compared to the first temperature-treated sample on the histogram (set to a value of 1), as described by the $\Delta\Delta C^T$ method (Livak & Schmittgen 2001) of transcript quantitation.

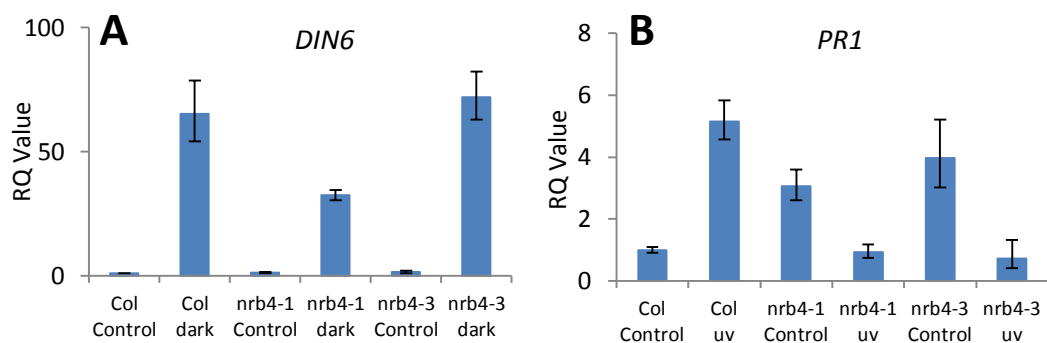


Figure 41: Relative quantification (RQ value) of dark- and UVC- inducible transcripts in response to dark or UV-C exposure. Cont: control 20°C conditions; dark: 6h darkness 3h after dawn; UV: 5KJ.m² UV-C. Error bars represent a 95% confidence interval from three technical repeats. Data is from one biological replicate experiment.

Preliminary results suggest that expression of dark-inducible gene, *DIN6* was impaired in the *nrb4-1* EMS mutant, while expression of UV-responsive gene *PR1* was impaired in both *nrb4* lines tested (Figure III.41). However, expression of *PR1* appeared to be upregulated in *nrb4* lines under untreated conditions compared to the wild type levels. The untreated condition of the seedlings (Figure III.40 & 41) was an unchanged 20°C temperature and light regime. The same set of seedlings acted as the control sample for cold, dark and UVC stresses.

III.8.2. CDK-8 domain

The CDK-8 domain of Mediator has been reported to be a regulatory domain that causes repression of gene activation when bound (Andrau *et al.*, 2006). It consists of 4 subunits: CDK8, CYCC, MED12 and MED13. Research has previously been carried out on the MED12 and MED13 subunits of the CDK-8 domain (Gillmor *et al.*, 2010, Imura *et al.*, 2012), but little is known about the CYCC and CDK8 subunits of the Mediator complex in plants.

III.8.2.1. PCR genotyping

III.8.2.1.1. CycC

T-DNA insertion lines from NASC that have not been previously published were tested for the presence of the T-DNA insert by conventional PCR and qRT-PCR was performed to show that reduced levels of full length transcript occurred in the mutant compared to the wild-type. Two different *CYCC* genes, *At5G48630* (*CYCC-1*) and *At5G48640* (*CYCC-2*), exist back-to-back in the Arabidopsis genome. A T-DNA insert line was available for each gene from NASC (SALK_039400 and SAIL_102_B02 respectively). As the genes are back-to-back within the Arabidopsis genome, creating a double mutant by conventional crossing as with *sfr6-1*, *med2-1* and *med14-1* would not be possible; therefore an RNAi approach may prove useful in the future as this was also not possible within the timeframe of the project.

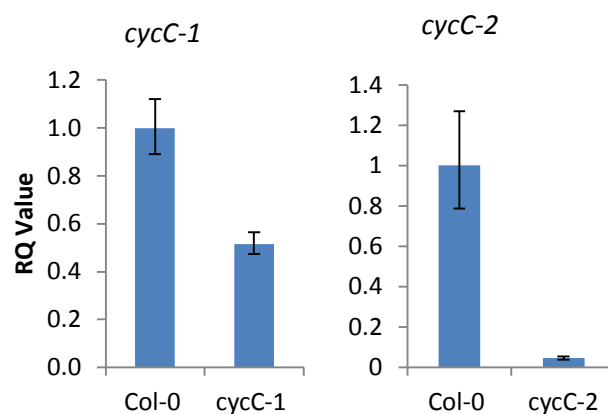


Figure 42: Relative quantification (RQ value) of transcript levels of *CYCC-1* (*At5g48630*, SALK_039400) and *CYCC-2* (*At5g48640*, SAIL_102_B02) under control conditions in wild type and *cycC-1* and *cycC-2* lines. Error bars represent a 95% confidence interval from 3 technical repeats. Data is from 2 biological replicate experiments.

The two NASC insert lines both contain T-DNA inserts in the promoter regions of the *CYCC1* (At5g48630) and *CYCC2* (At5g48640) genes. Data from qRT-PCR experiments in Figure III.42 (above) showed that transcript levels of *cycC-2* were reduced by at least 90% in the T-DNA insert line under normal and conditions. This suggests that the proposed *cycC-2* seed line identified from NASC can be considered a loss-of-function mutant. Transcript levels of *CYCC-1* were only reduced by 40% in the *cycC-1* insertion line, suggesting that the *cycC-1* seed line was not a complete loss-of-function mutant.

III.8.2.2. Phenotypes

III.8.2.2.1. CycC

There was no visible phenotype of the two *cycC* homozygous mutant lines, compared to wild type plants. This was potentially due to the two genes having redundant functions. No research has yet been published about *cycC* mutant lines in response to abiotic stresses although other CDK-8 domain subunits such as MED12 and MED13 have been investigated (Gillmor *et al.*, 2010, Imura *et al.*, 2012).

III.8.2.2.2. Cdk8

T-DNA insertion lines in the *CDK8* gene from NASC that have not been previously published were tested for disruption of the gene by conventional PCR to show the presence of the T-DNA insert in the proposed mutant line compared to the wild-type.



Figure 43: Electrophoresis gel showing the PCR product of the native *CDK8* gene Forward/Reverse primer pair used to identify *cdk8* homozygotes.



Figure 44: Photograph of the *cdk8* (At5g63610) homozygous T-DNA knock out (SALK_138675) plant before seed harvesting.

The phenotype for the *cdk8* homozygous mutant showed poor growth compared to wild-type *Arabidopsis thaliana* plants. *cdk8* plants only grew to 2/3 height and produced smaller leaves, had much fewer, smaller siliques that contained few seeds (Figure III.44) compared to wild type. The upper parts of the plants bore no siliques (as seen by Wang & Chen, 2004). The poor seed set of this *cdk8* mutant line meant that no further experiments on transcriptional regulation in response to abiotic stresses could be carried out in the time available.

III.8.2.3. Analysis of stress-induced gene expression in *cycC* mutants

Quantitative reverse transcriptase PCR (qRT-PCR) analysis was carried out on cold-, dark- and UV-inducible genes to assess relative expression levels of these genes in *cycC-1* and *cycC-2* lines compared to wild type.

III.8.2.3.1. Cold-treatment

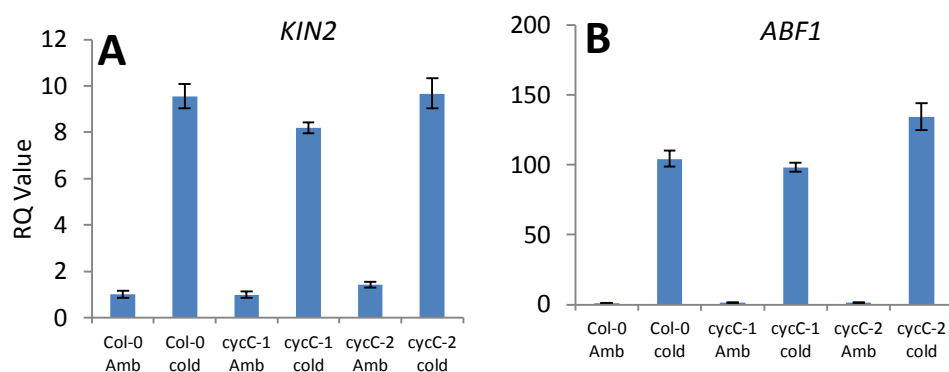


Figure 45: Relative quantification (RQ value) of cold-inducible transcripts in response to ambient or cold conditions. Cold: 6h at 5°C; Control: 6h at 20°C. Error bars represent a 95% confidence interval from 3 technical repeats. Data is from a single biological replicate experiment.

Preliminary experiments suggested that *cycC-1* and *cycC-2* mutants were not impaired in their transcriptional response to low temperatures compared to wild type. Further experimentation will be required to verify this result, however, it is likely that the T-DNA insert lines from NASC were not complete knock-out lines and still retained functionality when part of the Mediator complex resulting in normal gene expression compared to wild type.

III.8.2.3.2. UVC- & Dark-treatment

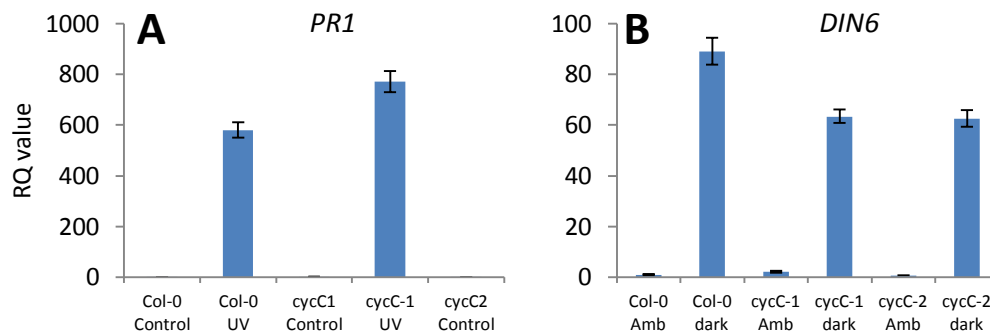


Figure 46: Relative quantification (RQ value) of UV-C and dark-inducible transcripts *PR1* and *DIN6* respectively. Amb: 20°C conditions; Dark: 6h darkness 3h after presumptive dawn; UV: 5KJ.m² UV-C. Error bars represent a 95% confidence interval of 3 technical repeats. Data is from one biological replicate experiment.

Preliminary data suggested that expression of UV-C-inducible gene, *PR1*, was not impaired in *cycC-1* or *cycC-2* lines (Figure III.46A). However, expression of dark-inducible gene *DIN6* appeared to be mildly impaired in both *cycC* mutant lines compared to the wild type (Figure III.46B). Further experiments would be required to confirm this result.

IV. Discussion

Previous research has identified a set of sensitive to freezing (*sfr*) Arabidopsis mutants including *sfr6* that could not survive freezing after a period of cold, non-freezing temperatures due to a failure to cold acclimate (Warren *et al.*, 1996). It was later discovered that in the case of *sfr6*, the failure to cold acclimate was due to a defect in *Cold On-Regulated (COR)* gene expression (Knight *et al.*, 1999). Knight went on to show that *sfr6* mutants specifically failed to express CBF-controlled *COR* genes in response to cold (Knight *et al.*, 2009). The SFR6 protein was later shown to be MED16, which forms part of a larger protein complex called Mediator (Bäckström *et al.*, 2007, Wathugala *et al.*, 2011) which interacts with both transcription factors and RNA pol II to mediate gene expression (Conaway & Conaway, 2011).

In light of previous research showing that several CBF-responsive genes were poorly expressed in response to cold in *sfr6* mutants (Knight *et al.*, 1999, 2009), microarray experiments were conducted to investigate whether all CBF-responsive genes were affected in this way and to ask whether loss of SFR6 affects cold-responsiveness of other genes that do not use CBFs. The work in this thesis validates the data obtained from the microarray experiments and tests whether all cold-inducible genes require the SFR6 subunit to be activated in response to cold. This study also expands on this idea, exploring the transcriptional response of cold-inducible genes in other Mediator subunit mutants, *med2*, *med14*, *med15*, *cycC1*, *cycC2* and *cdk8*. This is to investigate whether all subunits tested are required for correct cold-induced gene expression or if individual subunits play a specific role in the transcriptional response to abiotic stresses.

IV.1. Promoter motifs associated with genes that require SFR6 for cold-induction

Microarray data showed that not all cold-induced genes require the SFR6 subunit for their full expression. To further explore the role of SFR6 in the transcriptional regulation of cold-inducible genes, an analysis was carried out on 500 bp of the promoter regions of genes identified in microarray experiments as requiring SFR6 for their expression. ATHENA and RSAT analysis software was used to identify both known

and predicted transcription factor binding motifs to highlight potential differences between cold-inducible genes that are misregulated or non-misregulated in *sfr6*.

IV.1.1 The CRT/DRE motif

Using both the RSAT programme and the ATHENA software, a CCGAC sequence, which is the C-REPEAT/DROUGHT RESPONSE (CRT/DRE) motif, was found in the promoters of misregulated cold-inducible genes (Figure III.2). The CRT/DRE motif is a binding site of CBF transcription factors to induce expression of *Cold On-Regulated (COR)* genes that results in cold acclimation. CBFs binding to the CRT/DRE motif was first validated by *in vitro* experiments (Stockinger *et al.*, 1997, Liu *et al.*, 1998) and more recently *in vivo* by research carried out in our lab (Hemsley *et al.*, 2014) showing that SFR6 is required for all CBF-responsive gene expression.

IV.1.2 The EE motif

In addition to the CRT/DRE motif, the ATHENA software identified an AAAATATCA sequence, which is the full EVENING ELEMENT (EE), only in the group of cold-inducible genes that are misregulated in *sfr6* (Figure III.3). The EE is more commonly known to be involved in circadian clock signalling mechanisms than in the cold response (Carré & Kay, 1997), however data produced in this thesis is in accordance with previously published results (Mikkelsen & Thomashow, 2009) showing that the EE can be responsible for cold-inducibility in genes that do not contain a CRT/DRE motif and are not CBF-responsive. A link between *sfr6* mutants and impaired expression of EE-containing genes is unsurprising as an association between SFR6 and the EE-induced gene expression has previously been published (Knight *et al.*, 2008). However, the previously published association was in the context of *sfr6* and reduced expression of circadian clock components rather than abiotic stress.

The circadian clock is a signalling network that carries time-encoded information, an internal molecular clock that organisms use to measure time. It allows organisms to anticipate and respond to environmental changes on a daily and seasonal basis. The circadian clock can be divided into three parts: a central oscillator that generates inbuilt rhythmic behaviour in the plant, input pathways that carry environmental information to effect changes to the central oscillator and finally the output pathways

that regulate physiological processes as a result of changes to the central oscillator (Dunlap, 1999). The central oscillator is entrained by environmental time cues such as light, dark or temperature fluctuations (McClung, 2006) affecting transcriptional regulation feedback loops between the three different parts of the circadian clock. This can repress or activate transcription of clock-controlled genes to alter plant growth and development.

CIRCADIAN CLOCK ASSOCIATED1 (CCA1) (Wang & Tobin, 1998) and LATE ELONGATED HYPOCOTYL (LHY) (Schaffer, 1998), PSEUDORESPONSE REGULATOR (PRR) and TIMING OF CAB EXPRESSION1 (TOC1) (Millar, 1995; Makino, 2000) are four transcription factors that bind to the EE motif and act antagonistically to regulate transcription of clock-controlled genes (Alabadí *et al.*, 2001). Feedback loops between other unknown clock components and these transcription factors regulate expression of clock-controlled genes to alter plant development and growth in response to environmental signals (McClung, 2006). Expression of clock-controlled genes is also affected by phytohormones such as auxin (Covington & Harmer 2007). There is also evidence showing that the cold-inducibility of CBF-responsive transcript levels is gated by the circadian clock (Fowler *et al.*, 2005; Kreps *et al.*, 2002; Bieniawska *et al.*, 2008). Recent research has shown that CCA1 and LHY transcription factors are involved in the control of CBF expression, suggesting that the circadian clock does contribute to the process of cold acclimation (Dong *et al.*, 2011).

Expression of clock-controlled genes can also be affected by endogenous sugar levels (Blasing *et al.*, 2005). As presumptive evening approaches, environmental temperatures begin to fall. In C3 plants such as Arabidopsis, the combination of cooler temperatures and reduced light levels signals the approach of night and allows the plant to alter carbon metabolism pathways from photosynthesis and sugar storage to respiration and sugar usage (Geiger & Servaites, 1994). In this study the RSAT programme identified a SUGAR RESPONSE ELEMENT (SRE) as overrepresented in the promoters of the cold-inducible *sfr6* misregulated genes. The SRE sequence is AAATAT, a core sequence within the full EE motif, AAAATATCA (Figure III.3). This may explain the link between previous research showing that some clock-controlled genes are responsive to sugar (Blasing *et al.*, 2005). Interestingly, the expression of clock component genes is less affected by sugar levels in *sfr6* mutants than it is in wild type plants (Knight *et al* 2008).

IV.2. Temporal kinetics of cold-inducibility vary between cold-inducible genes

To further investigate the group of misregulated cold-inducible genes in *sfr6* identified from the microarray, the genes were divided into three subgroups depending on the promoter elements found 500 bp upstream of the predicted transcriptional start site of the gene. The three subgroups consisted of genes containing a CRT/DRE motif (CCGAC), an EE motif (AAAATATCA) or both motifs. A time course experiment was carried out under free-running conditions in wild type *Arabidopsis* under cold (5°C) and ambient (20°C) temperatures to investigate whether genes from each of the three subgroups were differentially expressed depending on the presumed time of day. It has been suggested that cold dampens oscillator cycles, disrupting expression of some circadian output genes (Gould, 2006; Bieniawska *et al.*, 2008). It has also been previously demonstrated that EE-containing genes are upregulated in the relative evening period (McClung, 2006).

The time course experiments described in this thesis revealed different patterns of relative transcript levels between the three subgroups of genes in wild type *Arabidopsis* but indicated an optimal time point of 6h at 5°C for significant levels of cold-induced gene expression in all three groups of genes, irrespective of their dynamics (Figure III.8). This time point was used in all further experiments. Genes that contained a CRT/DRE motif showed a continued increase in transcript levels over time (Figure III.11), with the exception of *GOLS3* (Figure III.11C) which showed an expression pattern similar to EE-containing genes (Figure III.10). This suggests that *GOLS3* expression in response to cold is not purely regulated by the presence of a CRT/DRE element but may also be affected by the presence of other promoter motifs such as the SRE, as *GOLS3* encodes an enzyme involved in galactinol synthesis (Taji *et al.*, 2001). Relative transcript levels of genes that contained the EE motif decreased after 9h of cold treatment (Figure III.10). This is possibly due to the effects of the circadian regulatory elements altering relative transcript levels rather than the cold-response as relative expression of EE-containing genes oscillated under ambient conditions during the time course (Figure III.8). Alternatively the EE may not provide a sustained response to cold, offering explanation of the oscillations seen in transcript levels during the timecourse. It is possible that while both the CRT/DRE and EE motifs

are cold-inducible, only the CRT/DRE motif provides a sustained response to cold, as seen in the transcript levels of CRT/DRE-containing genes (Figure III.8) while the EE provides a short-term response.

IV.2.1. The role of Mediator tail subunits in control of CBF-responsive CRT/DRE-containing genes

In light of the differences seen between CRT/DRE- and EE-containing genes in response to cold during the timecourse, relative transcript levels of misregulated genes that contained a CRT/DRE element, an EE element or both elements were then investigated after 6h at 5°C in plant lines where various Mediator subunits had been disrupted by either ethyl methanesulphonate (EMS) mutation (*sfr6-1*) (Knight *et al.*, 2009) or T-DNA insertion (*med2-1* and *med14-1* lines). This was to investigate whether other tail subunits are required for correct expression of these genes or whether this is a role specific to SFR6. In response to a treatment of 6h at 5°C, genes that contained a CRT/DRE promoter element showed reduced expression in *sfr6-1*, *med2-1* and *med14-1* seedlings compared to wild type (Figure III.11). This reduction could be due to a number of factors such as variations in mRNA processing, stability or turnover in response to cold temperatures. The reduced col-induced gene expression could also be due to impaired recruitment of transcriptional machinery such as Pol II or the Mediator Complex.

Chromatin immunoprecipitation (ChIP) experiments were therefore carried out to investigate the presence of transcriptional machinery at cold-inducible genes. ChIP experiments revealed that occupancy of Pol II was impaired in the three mutant lines under cold conditions compared to wild type levels along *KIN2*, *COR15A* and *LT178* (Figure III.16 & 18) genes, whose promoters contain the CRT/DRE promoter motif but no EE motif. A reduced occupancy of Pol II at cold-inducible genes would explain the reduced *COR* gene transcript levels and result in an inability to correctly cold acclimate and survive freezing conditions, as seen in *sfr6-1* mutants (Knight *et al.*, 1999). These data indicate that the SFR6 subunit and the MED2 and MED14 subunits are all required to recruit Pol II to cold-inducible promoters and express cold-inducible genes correctly.

Other current research in the lab (Hemsley *et al.*, 2014) suggests that recruitment of the whole Mediator complex to CRT/DRE-containing genes is also impaired in *sfr6-1*, *med2-1* and *med14-1* mutants compared to wild type in response to cold. Briefly, ChIP experiments were carried out against the MED6 (head submodule) subunit of the core Mediator complex and showed a reduced presence of core Mediator along the length of CRT/DRE-containing genes. This suggests that loss of SFR6, MED2 or MED14 subunits from the Mediator complex results in a reduced recruitment of both the Mediator complex and the RNA polymerase II enzyme. Research in the lab has also shown by ChIP experiments that CBF recruitment to the CRT/DRE motif in *GOLS3*, *LT178* and *KIN2* was normal in *sfr6-1* mutants in response to cold treatments. This suggests that CBF recruitment does not require the Mediator complex, but Pol II recruitment does. These data are in agreement with previous work carried out (Malik & Roeder 2010) showing that Mediator is recruited by transcription factors, followed by Pol II recruitment to the Mediator complex for gene activation. Therefore, poor recruitment and occupancy of vital transcriptional machinery such as the Mediator complex and by extension, Pol II along the length of cold-inducible genes is likely the cause of misregulation of cold-inducible genes that contain CRT/DRE promoter motifs.

Impairment of the SFR6, MED2 or MED14 subunits could result in a loss of transcription factor binding sites present on individual subunits or an incorrect conformation of the Mediator complex that does not promote transcription factor binding at promoter motifs. Individual subunits could play a structural role tethering subunits containing transcription factor binding sites onto the complex, which are lost as an indirect consequence of mutated tethering subunits. Cryo-electron tomography, a technique used to reconstruct 3D images from a series of 2D images, could prove an interesting tool to investigate Mediator conformation in *sfr6-1*, *med2-1* and *med14-1* plant lines compared to wild type Arabidopsis. This technique may reveal physical changes to the Mediator complex as a result of the *sfr6-1*, *med2-1* or *med14-1* mutations. Alternatively, the whole complex could be pulled down from mutant plants to see if various subunits are attached to the complex or whether they require the missing subunits in order to remain associated with the complex.

IV.2.1.1. The role of Mediator tail subunits in activation of expression via the CRT motif

Previously published work has shown that the CRT/DRE motif alone can respond to cold (Knight *et al.*, 2004). To further investigate the dependence of CRT/DRE-mediated cold-inducible expression upon these Mediator tail subunits, an artificial concatamer construct was expressed transiently in 7 day old *sfr6-1*, *med2-1* and *med14-1* seedlings. The data produced are in agreement with a previous study (Boyce *et al.*, 2003) where *sfr6* mutants expressed a CRT concatamer less than wild type in response to cold. Ideally stable transformant lines expressing the CRT/DRE::LUC+ concatamer in the *sfr6-1*, *med2-1* or *med14-1* backgrounds would form the best basis for analysis of the role of the CRT/DRE promoter element in response to various abiotic stresses such as cold. Stable transformant lines carrying both the concatamer and a Mediator subunit mutation have now been identified. Experiments with these lines were not possible in the time available for this project but will provide an interesting line of enquiry to future work.

IV.2.2. EE-containing genes do not show impaired Mediator and Pol II recruitment in *sfr6-1*, *med2-1* and *med14-1* in cold conditions

Amongst the genes shown in the microarray experiment to be cold-inducible were genes that contained an EE promoter element (AAATATCA) but no CRT/DRE motif. Previous work has demonstrated that the EE can confer cold-inducibility on some non-CRT/DRE genes (Mikkelsen & Thomashow, 2009). Experiments in this thesis showed that expression of EE-containing genes in response to cold is impaired in *sfr6-1*, *med2-1* and *med14-1* compared to wild type levels (Figure III.10), much like in the group of CRT/DRE-containing genes previously described. Expression of one EE-containing gene, At5g4470, was shown to be impaired in *sfr6-1* mutants, but not *med2-1* or *med14-1* lines. Other genes expressed to a similar level in the microarray were therefore investigated. Transcript data showed that none of the genes were also only misregulated in *sfr6-1* lines. This lends support to the idea that specific Mediator subunits are required for activation of certain cold-inducible genes. Unlike the CRT/DRE-containing genes however, recruitment of Pol II to EE-containing genes as shown by ChIP analysis (Figure III.17 & 19) is not impaired in *sfr6-1*, *med2-1* or *med14-1* compared to wild type in response to cold treatments (6h at 5°C).

Full length ChIP analysis of At1g20030 (a gene containing an EE but not CRT/DRE motif) shows that occupancy of Pol II along the full length of the gene is not increased in response to cold (Figure III.17) as seen in the CRT/DRE-containing genes. This could be explained by *de novo* recruitment of Pol II and transcription of CRT/DRE-containing genes such as *GOLS3* and *KIN2* in response to cold, while EE-containing genes are potentially constantly being transcribed in ambient conditions and modifications to the mRNA transcript that affect its stability or turnover occur in response to cold temperatures. Other current research has been carried out in the lab with ChIP experiments against the MED6 core Mediator subunit in EE-containing cold-inducible genes in wild type, *sfr6-1*, *med2-1* and *med14-1* mutants (Hemsley *et al.*, 2014). Results revealed that, like Pol II, occupancy of core Mediator is not impaired in any of the mutant lines under cold conditions compared to the occupancy seen in wild type seedlings. These data suggest that both Mediator and Pol II recruitment occurs correctly in EE-containing genes; however posttranscriptional modifications such as alternative splicing or mRNA transcript degradation may affect the number of detected transcripts (Hofmann, 2012; James *et al.*, 2012). Alterations to the mRNA may lead to an increase in transcript turnover due to altered poly-A tailing of the mRNA or alternative splicing events of the mRNA in response to cold (Chiba *et al.*, 2012). Alternatively spliced mRNA may potentially have been detected with the qRT-PCR primers used and misinterpreted as an increase in total transcript. Pol II occupancy may also not be a reflection of its activity; more Pol II is not recruited to EE-containing genes in response to cold, however it may have a greater activity in the cold that is dependent on the presence or absence of the subunits.

Experiments to investigate alternative splicing events in EE-containing genes were therefore carried out in wild type, *sfr6-1*, *med2-1* and *med14-1* under cold and ambient conditions. Data showed that alternate splice variants of the At1g20030 gene are not over or underrepresented in any of the mutants compared to wild type (Figure III.24). This suggests that for this gene, spliceosome activity is not impaired in the mutant lines and that the reduced transcripts of At1g20030 seen in *sfr6-1*, *med2-1* and *med14-1* are not due to an increased proportion of alternatively spliced or unspliced mRNA detected by the qRT-PCR primers. However, alternative splicing events in other EE-containing genes could be investigated. PolyA tailing of the transcripts could also be investigated to explore whether transcripts of At1g20030 and other cold-inducible

genes are degraded more rapidly in Mediator subunit lines compared to wild type (Gotic & Schilbler, 2013; Chiba *et al.*, 2012).

IV.2.3. Non-misregulated cold-inducible genes

A group of cold-inducible genes were also found not to be misregulated in *sfr6-1*, *med2-1* or *med14-1* mutants (Figure III.13), suggesting a level of cold-inducible gene activation that is independent of these three Mediator subunits, showing that certain Mediator subunits are required for some but not all cold-inducible genes. These genes were weakly induced in response to cold which may be the result of changes in transcript stability rather than activator-dependent transcription. However, further investigation into the activation mechanisms of these genes could prove interesting to elucidate the role of other transcriptional activation mechanisms, the role of other previously unknown Mediator subunits involved in the transcriptional response to cold or posttranscriptional modifications to the mRNA transcript in response to cold treatments.

IV.3. The SUGAR RESPONSE ELEMENT

A consensus sequence of AAATATC was found in the promoters of genes misregulated in *sfr6-1* mutants using the RSAT programme (Figure II.3). While it forms part of the full EE motif, it is also known to be the SUGAR RESPONSE ELEMENT (SRE) (Tatematsu *et al.*, 2005). The SRE was not found using the ATHENA software as it is not a known transcription factor binding sequence. However, previous research has shown that some clock-controlled genes are responsive to sugar (Blasing *et al.*, 2005). This is potentially because the SRE motif sequence forms the core of the full EVENING ELEMENT (AAAATATCA) promoter motif.

Sucrose is the major sugar synthesised in plants; during the day it is synthesised in the cytosol via photosynthesis and stored as a carbon store in the form of starch. At night the starch is degraded for the production of sucrose as an energy source (Fekke *et al.*, 2005). Sugars are important in plants as they serve as energy reserves, as building blocks for carbohydrate polymers such as starch or cellulose, as precursors of amino acids and as osmolytes which are necessary for the process of cold acclimation (Nägele *et al.*, 2010). Sugar accumulation has numerous roles in the cell, acting to reduce

cellular dehydration during freezing, helping proteins to maintain conformation and acts as an energy store (Levitt, 1980; Klotke *et al.*, 2004). It has also been suggested that sugars may contribute to cold acclimation in plants (Guy *et al.*, 1992) as overexpression of CBF3 causes freezing tolerance and the accumulation of sugars in *Arabidopsis* (Gilmour *et al.*, 2000). *sfr4*, another *sfr* mutant identified in the mutant screen with *sfr6* (Warren *et al.*, 1996) is also susceptible to freezing as it fails to accumulate sugars (Uemura *et al.*, 2003)

IV.3.1. Expression of sugar-response genes is impaired in *sfr6-1* and *med14-1* but not *med2-1* mutants

Research by Tatematsu (Tatematsu *et al.*, 2005) showed that the SRE and SRE-like motifs were found in genes repressed by sugar. However, results from this study showed that genes in wild type *Arabidopsis* containing both the SRE and a CRT/DRE promoter motif were upregulated in response to sucrose in wild type seedlings (Figure III.31A). Genes that did not contain a CRT/DRE motif were not upregulated in response to sucrose treatment. Experiments revealed that genes containing only the SRE and no CRT/DRE motif were more responsive to the presence of light rather than the presence of sucrose in wild type seedlings (Figure III.33). This could suggest that these genes are regulated more by light than sugar or that the changes in sugar levels caused by the light were sufficient to effect changes in sugar-responsive gene expression regardless of the addition or absence of extra sucrose to the media.

In light of the differential gene expression patterns seen as a result of the promoter elements present in wild type seedlings, expression of sugar-induced genes was investigated in *sfr6-1*, *med2-1* and *med14-1* mutants. This was to explore whether genes from the CRT/DRE and EE subgroups showed marked differences in patterns of transcript levels in response to sucrose as was seen in response to cold conditions.

Sugar-response experiments revealed that genes containing both a CRT/DRE motif and the SRE motif were differentially expressed in *sfr6-1*, *med2-1* and *med14-1* seedlings. Expression of genes that contained both CRT/DRE and SRE motifs were impaired only in *sfr6-1* and *med14-1* mutants in response to sugar and darkness (Figure III. 32, 33A & 34). Interestingly, these genes (*DIN6*, *AT1G20030*, *GOLS3* & *LT178*) were more highly expressed in *med2-1* than wild type seedlings in both experiments carried out (Figure

III.32 & 34). This could suggest that the MED2 subunit may function as a transcriptional repressor of sugar-response genes.

The increased sugar-induced gene expression in *med2-1* mutants implies a subunit-specificity in response to abiotic stresses: all three subunits investigated in this study are required for increased cold-induced transcript levels in response to cold, but only SFR6 and MED14 subunits are required for an increase in sugar- and dark-inducible gene transcript levels.

In response to sucrose, transcripts of *LT178* and *GOLS3* were impaired in *sfr6-1* and *med14-1* mutants (Figure III.34). Expression of *GOLS3* is likely to be affected and/or regulated by the presence of sucrose as it encodes an enzyme which is involved in synthesis of a carbohydrate, galactinol, a type of complex sugar, so its own expression could be regulated by sugar (Taji et al., 2001). However a similar pattern occurs in *LT178*, another CRT/DRE-containing gene, suggesting a general effect of sugar on CRT/DRE-containing genes rather than a *GOLS3*-specific effect. Transcriptional analysis of other CRT/DRE-containing genes identified in the microarray may provide further evidence to support this hypothesis.

It is also possible that other previously unidentified promoter elements are also present in these genes regulating their response to sugar that was not found in the RSAT or ATHENA promoter element analysis, but further analysis with different promoter programmes may be necessary to further investigate this. It is also possible that known cold-responsive genes could be prone to activation by sucrose via the CRT/DRE element as osmoprotectants often take the form of sugars and therefore an increase in the sugar content of the cell is synonymous of an osmotic stress to the plant, thus activating the CRT/DRE element in genes such as *LT178* and *GOLS3* (Asher & Schibler, 2011). Further investigation with other CRT/DRE-containing genes may prove useful in further validating this hypothesis.

IV.4. Additional Promoter motifs identified by RSAT in promoters of *sfr6*-misregulated genes

Using the RSAT analysis software and ATHENA programme, a CACGTG motif (Figure III.1) was also found to be overrepresented in the promoters of the 81 genes misregulated in *sfr6* and has been characterised as the abscisic acid response element (ABRE) (Mundy *et al.*, 1990). The ABRE is known to play a role in the activation of known cold-inducible genes (Haake *et al.*, 2002) by ABF and AREB transcription factors which are induced by the drought hormone, ABA (Narusaka *et al.*, 2003). Research shows that the ABRE acts as a coupling element for the EE (Mikkelsen & Thomashow 2009) and that salt- cold- and drought-stress responses share many common signalling networks in plants (Krasensky & Jonak, 2012). It is therefore unsurprising that the ABRE occurs at a greater frequency in cold-inducible genes than would normally be expected in the rest of the plant genome.

The RSAT analysis software also revealed numerous other motifs. Of these motifs, a CTTCTTC sequence was found (Figure III.4) that is known as a Y-patch, a pyrimidine-rich DNA sequence that has been implicated in transcriptional regulation of genes by DNA packing (Yamamoto *et al.*, 2007). Other motifs were also found present within 500 bp of the promoters of the non-misregulated gene group using the RSAT software that contained AAACCCTAAA, GCCCA and AAACAAA sequences (Figure III.6 & 7). These sequences have previously been found in other Arabidopsis genome-wide studies but have not yet been shown to have a role in DNA packing or acting as transcription factor binding sites in plants or animals (Mohanty *et al.*, 2005).

IV.5. Mediator Subunit Specificity

In light of the Mediator subunit requirement for the transcriptional responses to sucrose, dark (Figure III.32 & 34) and cold (Figure III.10 & 11), it would be interesting to investigate the contribution of each subunit to stress-induced transcript levels by assessing whether loss of two subunits causes more severe effects than loss of either singly. This would also elucidate whether *sfr6*, *med2* and *med14* had any overlapping redundant functions in abiotic stress response or if the roles they play are unique. Therefore crosses were made between *sfr6-1*, *med2-1* and *med14-1* mutants to create lines lacking expression of pairs of these subunits.

According to Mendelian genetics, as *SFR6*, *MED2* and *MED14* are on different chromosomes, it would be expected that 1/16 of the F₂ generation were homozygous for mutations on both genes. However, in genotyping these plant lines, it was found that a much smaller proportion of the population were homozygous double mutants, occurring at a frequency of 0.015 (*med2sfr6-1* and *med14sfr6-1* lines only), compared to the expected frequency of 0.063. It is therefore possible that this mutation was under negative selection bias due to reduced seed viability as a result of the mutation or a bias in the plants chosen to be tested for genotyping. To eliminate the latter possibility, an unbiased selection process was used in a second search for double mutant lines that successfully yielded double homozygous *med2-1sfr6-1* and *med14-1sfr6-1* mutants.

Due to the dwarfed phenotype and delayed flowering time of *med2-1sfr6-1* and *med14-1sfr6-1* (Figure III.38), it was not possible in the time available to carry out any abiotic stress assays on the progeny of these two plant lines. However, the dwarfed phenotype indicates some degree of additive or synergistic behaviour of *SFR6*, *MED2* and *MED14* as opposed to functional redundancy. One possibility is that activation of certain genes may require two different transcription factors that each bind different Mediator tail subunits or that binding sites of certain transcription factors spans two different subunits. It is also possible that one tail subunit may bridge other subunits containing the transcription factor binding site to the rest of the Mediator complex.. The current data could support any of these possibilities. Experiments with double Mediator subunit mutants will provide an interesting line of enquiry in future work to address these possibilities and further explore the function of the subunits in the transcriptional response to abiotic stresses investigated in this study.

IV.5. Additional Mediator Subunits investigated

IV.5.1. The MED15 subunit

Like the *SFR6*, *MED2* and *MED14* subunits, the *MED15* subunit is a predicted subunit in the tail domain of plant Mediator (Bourbon *et al.*, 2008, Gugliemi *et al.*, 2004). Research has shown that *med15* seedlings are profoundly insensitive to salicylic acid (SA), show compromised growth and the complete knock-out mutant is sterile, though other *med15* mutant lines are not. Research shows that the *MED15* subunit acts downstream of *NON-EXPRESSER OF PATHOGENESIS-RELATED GENE1* (*NPR1*) to

regulate the SA response in Arabidopsis (Canet *et al.*, 2012). Due to its predicted location adjacent to SFR6 in plant Mediator and the SA-responsive role it plays with MED16 (Wathugala *et al.*, 2012), it is possible that it also plays a role in the transcriptional response to other abiotic stresses as have been shown in MED2 and MED14 mutants in this study.

Three different *med15* mutant alleles were originally identified from an EMS mutant screen of plants insensitive to salicylic acid (Figure II.4). Seeds were sent later in the year and therefore the three *nrb4/med15* mutants were not part of the original cold responsive gene expression experiments.

In light of the transcriptional impairment seen in other tail subunits mutants (*sfr6-1*, *med2-1* and *med14-1*) in response to cold treatment and the proximity of the MED15 subunit to SFR6 in the plant Mediator complex, the three different alleles of *med15* seedlings (*nrb4-1*, *nrb4-2* and *nrb4-3*) were tested for transcriptional impairment in response to cold stress. Initial experiments revealed that the MED15 subunit may also be required for correct expression of CBF-regulated cold-inducible genes (Figure III.39). However, these data were found to be irreproducible in further experiments (Figure III.40).

As the MED2 subunit had shown differential involvement in the transcriptional response to cold, darkness and sugar, whereas MED16 is involved in all of these (, Knight *et al.*, 2009, Hemsley *et al.*, 2014) *nrb4-1* and *nrb4-3* alleles were subjected to UV-C stress and darkness to elucidate potentially unknown functions of the NRB4/MED15 subunit and to test whether it shares any other functions with the SFR6 subunit. Insufficient seeds of the *nrb4-2* allele meant that further analysis of stress-induced transcripts was not possible as it was for *nrb4-1* and *nrb4-3* alleles.

Preliminary experiments suggested that the *nrb4-1* allele had reduced transcripts of dark- (*DIN6*) and UVC- (*PR1*) responsive genes (Figure III.41). The *nrb4-3* allele showed reduced transcripts in response to UVC stress, but not in response to darkness compared to wild type (Figure III.41A). The *nrb4-1* and *nrb4-3* alleles showed reduced transcript levels of *PR1* in response to UV compared to wild type (Figure III.41B). Interestingly, *nrb4-1* and *nrb4-3* alleles also showed increased *PR1* transcripts under control conditions compared wild type which could suggest inappropriate gene activation either as a result of the mutation or a fungal contaminant on the growth

medium (Figure III.41B). Due to time constraints of the project, further research will be required in the future to further explore and validate this result.

IV.5.2. CDK-8 Domain

Early literature on Mediator suggested that the CDK-8 domain of the Mediator complex plays a negative regulatory role on transcription in response to abiotic stress in yeast by its association or dissociation with the rest of the Mediator complex (Andrau *et al.*, 2006, Gonzalez *et al.*, 2007). Much work has been carried out in plants on two of the four subunits of the CDK-8 domain, MED12 and MED13, implicating them in negative regulation of transcription during embryo patterning (Gillmor *et al.*, 2010) and flower formation (Imura *et al.*, 2012). Other work on the CDK8 subunit of the CDK-8 domain has shown that *cdk8* mutants have impaired floral organ formation and seed set (Wang & Chen, 2004), a phenotype also seen in this study (Figure III.45).

Experiments in yeast and human cells have shown that the CDK-8 domain has a transient interaction with core Mediator as it is recruited in a gene-specific manner to the core Mediator complex to repress transcription of certain genes in response to abiotic stress (Andrau *et al.*, 2006; Donner, 2010; Knuesel, 2009). Recently, research in plants provides evidence to suggest that CDK8 plays a dual role integrating cellular responses to environmental signals to promote growth and act as a relay between stress-induced transcription factors and Pol II (Ng *et al.*, 2012). This offers an explanation to opposing research showing CDK8 as both a transcriptional repressor (Andrau *et al.*, 2006) and a transcriptional activator (Ng *et al.*, 2012) depending on the environmental conditions.

Little is known of the functions of the last subunit of the CDK-8 domain, CYCC, and whether it also functions as a negative regulator of transcription. Preliminary experiments in this study suggest that two back-to-back genes in the same orientation both encode the CYCC subunit of the plant Mediator complex and that the *cycC* responses to cold and UV-C stress are unimpaired in both T-DNA insert lines tested (Figure III.45 & 46A). Despite this, preliminary experiments show that transcripts of the dark-inducible gene *DIN6* are reduced in both *cycC* alleles compared to the wild type (Figure III.46B), suggesting a role for the CYCC subunit in the expression of dark-

inducible genes in plants. This could suggest that the *CYCC-2* gene is the functional one while *CYCC-1* plays a more redundant role.

More recently, evidence is emerging to counteract the theory that the CDK-8 domain is a general negative transcriptional regulator (Ng et al., 2012). Indeed, preliminary results in this study show that transcription is not generally repressed in *cycC* lines in response to cold and UV-C stress despite the impaired response to darkness (Figure III.46B). This lends weight to the argument that different sets of Mediator subunits from all 4 domains are required to for full expression of different sets of abiotic stress-response genes in Arabidopsis. However, as the *CYCC* subunit is encoded by two genes, functional redundancy may account for the normal transcriptional response to cold and UVC stress seen in this study. In light of this, creation of a double *cycC* mutant would allow a fuller investigation into the role played by the *CYCC* subunit in transcriptional regulation. As *CYCC-1* and *CYCC-2* are back-to-back genes in the Arabidopsis genome, it would be difficult to create a double mutant line by conventional crossing. RNAi would prove a more useful technique for disruption of the *CYCC-1* gene in *cycC-2* lines and allow transcriptional analysis of genes in response to abiotic stresses when expression of both genes is impaired.

IV.6. Conclusions & Future Work

This study shows that MED2 and MED14 control an overlapping set of cold-inducible genes with SFR6. Future structural studies on the plant Mediator complex, as have been carried out in yeast (e.g. Gugliemi 2004), may explain the differential involvement of various subunits in the response to abiotic stresses. This will enable evaluation of the nature of the contribution (structural and conformational changes, transcription factor binding sites and interactions) SFR6, MED2 and MED14 subunits play in abiotic stress-induced gene activation. Mediator mutants stably expressing a CRT/DRE::LUC reporter may elucidate the interaction of different Mediator subunits with the CRT/DRE in cold-inducible expression.

This study demonstrated that reduced transcripts of cold-inducible genes in *sfr6-1*, *med2-1* and *med14-1* are due to an impaired Pol II recruitment to genes that contain CRT/DRE promoter motifs. Pol II recruitment to genes that contained the EE promoter motif was not impaired, suggesting a Pol II-independent mode of gene activation or a

role for unknown post-transcriptional modifications to the mRNA transcripts or a post recruitment role. Further work on identifying alternatively spliced mRNA transcripts of EE-containing genes could provide further illumination on activation of EE-containing gene transcripts in the process of cold acclimation.

Research in this study has also shown the previously unknown effect of sucrose and light on CRT/DRE- and EE-containing cold-inducible genes in wild type plants and its dependency on the SFR6/MED16, MED2 and MED14 subunits. Experiments revealed differential involvement of the MED2 subunit in activation of genes in response to sucrose compared to the SFR6 and MED14 subunits. Preliminary experiments with another predicted tail subunit mutant, *nrb4/med15*, suggested that the NRB4/MED15 subunit does not play a role in the transcriptional response to low temperature but may be involved in the response to UV-C and darkness.

Preliminary results suggest that the CDK-8 domain may not act as a general transcriptional repressor as was previously assumed (Gonzalez *et al.*, 2007) *cycC* mutants were only mildly affected in the transcriptional response to darkness but not cold or UV-C stress. Future work will be required to gain a fuller understanding of the role played by the 4 subunits of the CDK-8 domain in transcriptional regulation. Structural studies and co-immunoprecipitation experiments with subunits of the CDK-8 domain could reveal previously unknown interactions with the rest of the Mediator complex and shed light on the much-debated role it plays in transcriptional regulation.

Although this study was limited to a small number of Mediator subunits, it begins to illustrate that transcriptional regulation in response to different abiotic stresses occurs as a result of the cooperation between different sets of subunits within the Mediator complex. Future work with other single and double Mediator subunit mutants will highlight the role each subunit plays in the transcriptional response to abiotic stress.

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Appendix I: Buffers

TE Buffer:

10mM Tris-HCL pH8.0, 5mM EDTA pH7.5

Edward's Extraction buffer:

200mM Tris-HCL pH 7.5, 250mM NaCl, 25mM EDTA pH 8.0, 5% v/v SDS

ChIP Buffers

Extraction Buffer 1

0.4M sucrose, 10mM Tris-HCL pH8.0, 10mM MgCl₂, 1% v/v Triton X-100, 5mM β-mercaptoethanol, 0.35% v/v protease inhibitor (SIGMA protease inhibitor cocktail P-9599)

Extraction Buffer 2

0.25M sucrose, 10mM Tris-HCL pH8.0, 10mM MgCl₂, 1% v/v Triton X-100, 5mM β-mercaptoethanol, 0.35% v/v Protease inhibitor

Nuclear Lysis Buffer

10mM Tris-HCL pH8.0, 150mM NaCl, 1mM EDTA pH8.0, 0.1% v/v Sodium deoxycholate, 0.5% v/v sarcosine, 0.35% v/v protease inhibitor

ChIP Dilution Buffer

10mM Tris-HCL pH8.0, 150mM NaCl, 1mM EDTA, 1% v/v Triton X-100, 0.35% v/v protease inhibitor

Low Salt Wash Buffer

150mM NaCl, 0.1% SDS, 1% v/v Triton X-100, 2mM EDTA, 20mM Tris-HCL pH8.0

High Salt Wash Buffer

500mM NaCl, 0.1% v/v SDS, 1% v/v Triton X-100, 2mM EDTA, 20mM Tris HCL pH8.0

LiCl Wash Buffer

0.25M LiCl, 1% v/v NP-40, 1% v/v sodium deoxycholate, 1mM EDTA, 10mM Tris-HCL pH8.0

TE RNaseA

10mM Tris-HCL pH8.0, 1mM EDTA, 10μg.mL⁻¹ RNaseA

Appendices

Gel electrophoresis

TBE Buffer

A stock of 0.5X TBE was made using 45mM tris-borate, 1mM EDTA pH 8.0 for use in the agarose gel and running buffer.

Luciferase

Spray

A 100mM stock of luciferin diluted to 5mM in a 1% v/v Triton-X 100 solution.

Liquid

A 100mM stock of luciferin diluted to 1mM in ddH₂O.

Protoplast extraction & transformation

Enzyme Solution

1% w/v cellulose, 0.25% w/v maceroenzyme, 0.5M mannitol, 8mM CaCl₂ adjusted to pH5.5 and filter sterilised through a 0.45µm cellulose filter [VWR 28145-477].

Resuspension Buffer I (RB I)

30ml 0.5M mannitol, 60ml 0.2M CaCl₂

Resuspension Buffer II (RB II)

60ml 0.5M mannitol, 30ml 0.2M CaCl₂

W5 solution

154mM NaCl, 125mM CaCl₂, 5mM KCl, 5mM glucose, 1.5mM MES-KOH pH5.6

Mannitol/Mg solution

15mM MgCl₂, 0.1% v/v MES, 0.4M mannitol adjusted to pH5.6 with 0.1MKOH and autoclaved

PEG-CMS solution

0.4M mannitol, 100mM calcium nitrate, 40% w/v PEG 4000

Protoplast culture medium

400mM sucrose, 1xMS salts, 250mg/L xylose adjusted to pH 5.8 with 0.1M KOH and autoclaved.

Appendices

Mannitol/W5 solution (M/W5 solution)

400mM mannitol diluted 1 in 4 with W5 solution

Appendix II: Lists of 81 cold-inducible misregulated and non-misregulated genes

81 cold-inducible misregulated genes

These genes showed at least a 1.5-fold induction in wild type plants compared to ambient in microarray experiments (Affymetrix gene chip GEO reference:GSE6167).

Wild type (WT) and *sfr6-1* gene expression data is from the Affymetrix gene chip GEO reference GSE6167. The *sfr6-2* and *sfr6-3* data is from the Affymetrix gene chip GEO reference GSE46084. A gene was said to be misregulated in *sfr6* if the ratio of all three *sfr6* alleles (*sfr6-1*, *sfr6-2* and *sfr6-3*) to wild type was less than 0.7, i.e. these genes showed a minimum 30% reduction in gene expression in all 3 *sfr6* alleles compared to wild type.

Gene	Accession	Description	WT1 cold	<i>sfr6-1</i> cold	<i>sfr6-1</i> /WT1 Ratio	WT2 cold	<i>sfr6-2</i> cold	<i>sfr6-2</i> /WT2 Ratio	WT3 cold	<i>sfr6-3</i> cold	<i>sfr6-3</i> /WT3 ratio
UDP-glucuronosyl/UDP-glucosyl transferase family protein	At1g01420	flavonol 3-o-glucosyltransferase, putative similar to flavonol 3-o-glucosyltransferase GB:Q40287 from [Manihot esculenta]	291.872	204.003	0.6989468	613.355	199.848	0.32582762	740.617	278.259	0.375712413
galactinol synthase, putative	At1g09350	putative galactinol synthase similar to GB:AAD26116 from [Brassica napus]; supported by cDNA: gi_13899102_gb_AF370546.1_AF370546	615.518	190.192	0.30899503	1796.926	56.044	0.03118882	4522.038	788.74	0.17442135
cytochrome P450 family protein	At1g12740	cytochrome P450, putative similar to cytochrome P450 Gl:4176420 from [Arabidopsis thaliana]	194.247	102.149	0.5258717	73.51	39.295	0.53455312	222.415	103.025	0.46321066
pathogenesis-related thaumatin family protein	At1g20030	calreticulin, putative similar to GB:AAF06346 from [Vitis vinifera]	1104.569	546.643	0.49489258	490.285	147.094	0.30001734	2006.977	915.973	0.45639436
dehydrin (ERD10)	At1g20450	hypothetical protein ; supported by cDNA: gi_15081631_gb_AY048208.1_	4628.388	2256.994	0.48764149	10728.304	4174.608	0.38912096	13822.834	7796.736	0.56404757
expressed protein	At1g24575	Expressed protein ; supported by full-length cDNA: Ceres: 38093.	138.525	84.135	0.60736329	101.17	45.783	0.45253534	108.092	23.461	0.21704659
zinc finger (C2H2 type) family protein (ZAT10) /	At1g27730	salt-tolerance zinc finger protein identical to salt-tolerance zinc finger protein GB:CAA64820	859.43	410.399	0.47752464	734.548	158.103	0.21523849	422.852	51.813	0.12253223

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salt-tolerance zinc finger protein (STZ)		GI:1565227 from [Arabidopsis thaliana]; supported by cDNA: gi_14334649_gb_AY034998.1_									
glycosyl hydrolase family 17 protein	At1g32860	beta-1,3-glucanase precursor, putative similar to beta-1,3-glucanase precursor GI:4097948 from [Oryza sativa]	502.37	245.042	0.48777196	222.072	98.996	0.44578335	1628.257	254.829	0.15650416
expressed protein	At1g33230	unknown protein ;supported by full-length cDNA: Ceres:8976.	573.56	357.04	0.62249808	744.6	359.215	0.48242681	1593.627	433.117	0.27178066
serpin, putative / serine protease inhibitor, putative	At1g47710	serpin, putative similar to serpin GB:X95277 GI:1197576 from (Hordeum vulgare)	1281.314	595.141	0.46447709	2109.753	581.58	0.2756626	4769.654	963.071	0.20191632
glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	At1g48100	polygalacturonase PG1, putative similar to GB:AAD46483 from [Glycine max] (Mol. Plant Microbe Interact. 12 (6), 490-498 (1999)); supported by cDNA: gi_15292728_gb_AY050798.1_	401.876	137.794	0.34287691	1403.612	114.239	0.0813893	2790.533	170.151	0.06097437
ABA-responsive element-binding protein / abscisic acid responsive elements-binding factor (ABRE)	At1g49720	abscisic acid responsive elements-binding factor identical to abscisic acid responsive elements-binding factor GB:AAF27179 GI:6739274 from [Arabidopsis thaliana]; supported by cDNA: gi_6739273_gb_AF093544.1_AF093544	1532.98	1025.379	0.66887957	1171.78	483.029	0.41221816	2137.028	1172.583	0.54869800
heavy-metal-associated domain-containing protein	At1g51090	proline-rich protein, putative similar to proline-rich protein GI:3242079 from [Capsicum annuum]; supported by cDNA: gi_14334847_gb_AY035097.1_	573.723	221.263	0.38566172	1134.879	150.556	0.1326626	3979.105	898.575	0.22582339
expressed protein	At1g53035	Expressed protein ; supported by full-length cDNA: Ceres: 271765.	1754.213	968.068	0.55185317	493.646	119.349	0.24177042	2083.381	829.906	0.39834576
integral membrane transporter family protein	At1g64890	unknown protein ; supported by cDNA: gi_15028136_gb_AY046018.1_	773.646	246.505	0.31862764	271.666	98.306	0.36186346	1150.62	268.486	0.23334028
expressed protein /// expressed protein	At1g66000	unknown protein	89.751	53.188	0.59261735	180.659	58.227	0.32230334	192.093	62.093	0.32324447
expressed protein	At1g68500	hypothetical protein predicted by genefinder	1226.004	156.196	0.12740252	2340.24	147.8	0.06315592	3684.59	275.263	0.07470654
expressed protein	At1g69760	unknown protein ;supported by full-length cDNA: Ceres:18367.	1001.895	633.758	0.6325593	1435.111	491.716	0.34263273	1299.205	492.491	0.37907104
hydrolase, alpha/beta fold family protein	At1g73480	lysophospholipase homolog, putative similar to lysophospholipase homolog GI:2801536 from [Oryza sativa]; supported by cDNA: gi_15028212_gb_AY045929.1_	1253.427	657.632	0.52466717	2694.336	719.834	0.26716564	3319.905	1111.222	0.33471499

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expressed protein	At1g75860	unknown protein	882.47	433.242	0.49094247	712.672	264.602	0.3712816	1117.141	385.064	0.34468701
SPL1-Related3 protein (SPL1R3)	At1g76580	unknown protein similar to SPL1-related proteins: GB:CAB56771, GB:CAB56770, GB:CAB56773 [Arabidopsis thaliana]	270.181	161.004	0.59591163	325.208	114.061	0.35073245	632.893	163.161	0.25780187
zinc-binding family protein	At1g76590	unknown protein ;supported by full-length cDNA: Ceres:114376.	691.824	272.538	0.39394123	1448.001	170.007	0.11740807	1345.896	294.569	0.21886460
alcohol dehydrogenase (ADH)	At1g77120	alcohol dehydrogenase identical to alcohol dehydrogenase GI:469467 from (Arabidopsis thaliana); supported by full-length cDNA: Ceres: 4033.	761.11	491.928	0.6463297	775.673	402.574	0.51899963	3707.75	2103.285	0.56726721
expressed protein	At1g79660	predicted protein	1048.567	503.721	0.4803899	4774.979	457.462	0.09580398	3276.783	451.785	0.13787455
senescence/dehydration-associated protein-related (ERD7)	At2g17840	putative senescence-associated protein 12 ;supported by full-length cDNA: Ceres:40806.	1340.403	517.19	0.38584664	1664.822	275.225	0.16531797	5325.376	1056.251	0.19834299
expressed protein	At2g19390	unknown protein ; supported by cDNA: gi_16604626_gb_AY059758.1_	46.311	26.69	0.57632096	94.54	38.61	0.40839856	91.074	53.435	0.58672068
glycosyl hydrolase family 17 protein /// glycosyl hydrolase family 17 protein	At2g19440	putative beta-1,3-glucanase	378.414	221.606	0.58561787	175.804	119.503	0.67975131	286.649	153.484	0.53544230
expressed protein	At2g22080	En/Spm-like transposon protein related to En/Spm transposon family of maize	415.173	264.167	0.63628174	1143.629	297.322	0.25998117	1290.442	560.711	0.43451081
AP2 domain-containing transcription factor, putative	At2g23340	putative AP2 domain transcription factor	719.989	322.401	0.44778601	671.968	138.307	0.20582379	2423.684	540.687	0.22308477
mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	At2g28900	putative membrane channel protein ;supported by full-length cDNA: Ceres:10159.	1905.727	1123.82	0.58970671	5538.4	1926.291	0.34780641	7219.076	4130.066	0.57210451
cytochrome P450 family protein	At2g29090	putative cytochrome P450	137.65	39.692	0.28835452	148.147	62.936	0.42482129	60.062	24.641	0.41025939
expressed protein	At2g36220	unknown protein ;supported by full-length cDNA: Ceres:12251.	1045.746	620.712	0.59355905	1199.315	427.86	0.35675365	1921.956	676.011	0.35173073
expressed protein	At2g39705	Expressed protein ; supported by full-length cDNA: Ceres: 27620.	580.366	301.115	0.51883639	400.505	147.999	0.36953097	689.737	228.704	0.33158145
acid phosphatase class B	At2g39920	hypothetical protein predicted by	507.991	240.465	0.47336469	139.704	63.229	0.45259262	447.097	294.471	0.65862888

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family protein		genscan;supported by full-length cDNA: Ceres:33701.									
cold-responsive protein / cold-regulated protein (cor15b)	At2g42530	cold-regulated protein cor15b precursor ;supported by full-length cDNA: Ceres:19221.	2332.839	1147.021	0.4916846	5387.203	1467.117	0.27233371	5224.701	2517.639	0.48187236
cold-responsive protein / cold-regulated protein (cor15a)	At2g42540	cold-regulated protein cor15a precursor ; supported by cDNA: gi_14532457_gb_AY039853.1	2134.961	633.755	0.29684617	8821.425	1649.688	0.18700924	10009.884	3562.287	0.35587695
MADS-box protein (AGL20)	At2g45660	MADS-box protein (AGL20) ; supported by full- length cDNA: Ceres: 5467.	257.743	150.79	0.58504014	1191.943	253.684	0.21283233	856.617	318.402	0.37169703
expressed protein	At3g05800	hypothetical protein predicted by genscan+;supported by full-length cDNA: Ceres:21672.	1743.748	1218.685	0.69888826	497.27	208.052	0.4183884	1848.854	797.047	0.43110326
pectate lyase family protein	At3g09540	putative pectate lyase simliar to pectate lyase precursor GB:P40972 from [Nicotiana tabacum]	255.223	169.371	0.66361966	322.337	111.643	0.3463549	629.357	239.122	0.37994651
DNAJ heat shock N- terminal domain- containing protein /// DNAJ heat shock N- terminal domain- containing protein	At3g13310	DnaJ protein, putative contains Pfam profile: PF00226 DnaJ domain;supported by full-length cDNA: Ceres:31309.	1267.005	281.963	0.22254293	1375.822	209.914	0.15257352	826.735	242.835	0.29372773
ethylene-responsive element-binding factor 4 (ERF4)	At3g15210	ethylene responsive element binding factor 4 (AtERF4) identical to GB:BAA32421 from [Arabidopsis thaliana];supported by full-length cDNA: Ceres:22775.	1191.188	498.393	0.41839995	1441.647	564.856	0.39181298	994.117	331.601	0.33356335
invertase/pectin methylesterase inhibitor family protein	At3g17130	hypothetical protein predicted by genemark.hmm	369.142	171.406	0.46433622	459.403	66.314	0.14434821	1552.657	130.412	0.08399279
expressed protein	At3g23170	unknown protein ;supported by full-length cDNA: Ceres:92314.	1235.798	676.743	0.5476162	214.107	90.227	0.42141079	557.033	165.197	0.29656591
expressed protein	At3g25870	unknown protein ;supported by full-length cDNA: Ceres:141813.	226.211	21.928	0.09693605	122.056	17.191	0.14084519	405.037	38.873	0.09597394
expressed protein	At3g27880	hypothetical protein predicted by genemark.hmm;supported by full-length cDNA: Ceres:15282.	552.206	352.114	0.63764972	167.386	100.842	0.60245182	652.975	397.194	0.60828362
cytochrome P450 family protein	At3g44970	cytochrome P450 - like protein cytochrome P450 d13695c, Arabidopsis thaliana, PIR:C71417	625.969	303.801	0.48532915	623.645	254.421	0.40795805	531.852	210.309	0.39542767
dehydrin xero2 (XERO2) /	At3g50970	dehydrin Xero2 ; supported by cDNA:	2638.137	636.637	0.24132067	12680.454	2522.688	0.19894303	14551.283	4983.044	0.34244705

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low-temperature-induced protein LTI30 (LTI30)		gi_15809983_gb_AY054260.1_									
expressed protein	At3g51750	hypothetical protein	141.339	58.314	0.41258251	74.798	35.114	0.46945105	83.665	23.141	0.27659116
alkaline alpha galactosidase, putative	At3g57520	imbibition protein homolog probable imbibition protein - Brassica oleracea, PIR:S45033; supported by cDNA: gi_15292676_gb_AY050772.1_	1734.538	1160.978	0.66932982	8632.648	4599.374	0.53278832	1325.846	766.035	0.57777072
expressed protein	At3g60520	putative protein ;supported by full-length cDNA: Ceres:21518.	1110.828	610.97	0.55001314	528.513	221.928	0.4199102	861.46	380.924	0.44218419
BON1-associated protein 1 (BAP1)	At3g61190	putative protein hypothetical protein F4I18.26 - Arabidopsis thaliana, PIR:T02471;supported by full-length cDNA: Ceres:30454.	360.374	176.982	0.49110646	735.589	51.711	0.07029877	403.197	53.54	0.13278868
CBL-interacting protein kinase 4 (CIPK4)	At4g14580	SNF1 like protein kinase ; supported by cDNA: gi_13249502_gb_AY007221.1_	690.32	469.907	0.68070895	99.559	54.168	0.54407939	408.201	239.964	0.58785745
glycosyl transferase family 4 protein	At4g18270	putative protein phospho-N-acetylmuramoyl-pentapeptide-transferase, Haemophilus influenzae,Pir2:A64185	695.254	445.824	0.64123903	262.704	153.981	0.58613877	651.186	190.578	0.29266292
glycine-rich cell wall protein-related	At4g18280	glycine-rich cell wall protein-like glycine-rich protein 1.0 precursor, Phaseolus vulgaris, PIR1:S01821; supported by cDNA: gi_14030676_gb_AF375429.1 AF375429	523.97	234.027	0.44664198	367.764	79.387	0.21586398	1908.111	326.103	0.17090358
iron-responsive transporter (IRT2)	At4g19680	putative Fe(II) transport protein Fe(II) transport protein, Arabidopsis thaliana, gb:U27590	183.664	95.016	0.517336	64.002	36.516	0.57054467	124.929	56.05	0.44865483
ABA-responsive protein (HVA22d)	At4g24960	abscisic acid-induced - like protein abscisic acid-induced protein HVA22, Hordeumvulgare, PIR2:A48892;supported by full-length cDNA: Ceres:28535.	1320.8	370.519	0.2805262	4262.871	574.2	0.13469795	5965.98	1157.281	0.19398003
glycosyl hydrolase family 1 protein	At4g27820	putative beta-glucosidase beta-glucosidase BGQ60 precursor - barley, PIR2:A57512	1250.489	764.867	0.61165432	1356.701	912.828	0.67282916	3090.573	916.427	0.29652333
zinc finger (CCCH-type) family protein	At4g29190	putative protein zinc finger transcription factor - Arabidopsis thaliana,PID:g2961542;supported by full-length cDNA: Ceres:16432.	2184.6	1331.93	0.60969056	3444.45	1112.481	0.32297783	2609.259	1258.334	0.48225722
hydrophobic protein, putative / low temperature and salt responsive protein, putative	At4g30650	low temperature and salt responsive protein homolog low temperature and salt responsive protein LTI6A - Arabidopsis thaliana,PID:g4039153	1898.811	920.6	0.48482972	2739.773	601.608	0.21958316	3464.831	1863.086	0.53771338
pyruvate decarboxylase,	At4g33070	pyruvate decarboxylase-1 (Pdc1)	216.101	135.108	0.62520766	357.04	78.28	0.21924714	2190.617	772.068	0.35244317

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putative											
zinc finger (C3HC4-type RING finger) family protein	At4g35480	RING-H2 finger protein RHA3b ;supported by full-length cDNA: Ceres:31493.	644.387	402.729	0.62498002	544.605	218.432	0.40108335	797.618	360.626	0.45212871
pathogenesis-related thaumatin family protein	At4g36010	thaumatin-like protein thaumatin-like protein, Arabidopsis thaliana, Pir2:S71175; supported by cDNA: gi_13430505_gb_AF360165.1_AF360165	2764.088	1094.356	0.39591938	830.607	136.36	0.1641691	3234.546	825.364	0.25517151
expressed protein	At4g36500	putative protein	2230.408	1255.688	0.56298579	2321.841	609.377	0.26245423	2050.377	739.348	0.36059124
heavy-metal-associated domain-containing protein / copper chaperone (CCH)-related	At4g38580	farnesylated protein (ATFP6) ; supported by full-length cDNA: Ceres: 10238.	909.445	432.005	0.47502048	3596.06	451.003	0.12541587	4415.486	934.62	0.21166865
zinc finger (C2H2 type) family protein	At5g04340	putative c2h2 zinc finger transcription factor	613.972	262.987	0.42833712	2083.316	162.561	0.07802993	1198.959	263.593	0.21985155
stress-responsive protein (KIN1) / stress-induced protein (KIN1) /// stress-responsive protein (KIN1) / stress-induced protein (KIN1)	At5g15960	cold and ABA inducible protein kin1 ;supported by full-length cDNA: Ceres:2270.	3152.109	610.111	0.19355644	14067.651	3189.086	0.22669641	14222.241	3892.383	0.27368281
expressed protein	At5g19875	Expressed protein ; supported by full-length cDNA: Ceres: 59.	534.017	223.1	0.41777696	520.824	107.778	0.20693747	725.614	109.229	0.15053320
expressed protein	At5g22390	unknown protein	288.328	190.027	0.65906537	578.57	323.01	0.55829027	255.119	160.952	0.63088989
prephenate dehydratase family protein	At5g22630	chorismate mutase/prephenate dehydratase-like protein ; supported by cDNA: gi_16604397_gb_AY058097.1_	1050.414	722.282	0.6876165	400.462	253.865	0.63393031	1236.448	854.117	0.69078279
proline oxidase, putative / osmotic stress-responsive proline dehydrogenase, putative	At5g38710	proline oxidase, mitochondrial precursor -like protein PROLINE OXIDASE, MITOCHONDRIAL PRECURSOR, Arabidopsis thaliana, SWISSNEW:PROD	187.707	92.46	0.4925762	242.919	38.528	0.15860431	78.248	53.322	0.68144872
reticulon family protein (RTNLB4)	At5g41600	putative protein contains similarity to 24 kDa seed maturation protein; supported by cDNA: gi_14334529_gb_AY035169.1_	1155.581	798.068	0.69062056	1345.917	505.34	0.37546149	1389.092	760.263	0.54730932
zinc-binding family protein	At5g46710	putative protein similar to unknown protein (pir T05076);supported by full-length cDNA: Ceres:42747.	1442.876	814.059	0.56419193	1212.831	433.153	0.35714209	1449.837	305.334	0.21059884
ethylene-responsive element-binding factor 5	At5g47230	ethylene responsive element binding factor 5 (ATERF5) (sp O80341) ; supported by cDNA:	347.018	214.689	0.6186682	87.507	39.036	0.44609003	256.197	86.003	0.33569089

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(ERF5)		gi_14326511_gb_AF385709.1_AF385709									
zinc finger (B-box type) family protein	At5g48250	putative protein contains similarity to CONSTANS homologs	1248.126	708.851	0.56793224	669.667	331.75	0.49539547	1683.239	988.827	0.58745490
33 kDa secretory protein-related	At5g48540	33 kDa secretory protein-like ; supported by cDNA: gi_15292980_gb_AY050924.1_	825.885	529.17	0.64073085	762.205	183.545	0.24080792	1225.539	323.704	0.26413194
acetyl-CoA C-acyltransferase 1 / 3-ketoacyl-CoA thiolase 1 (PKT1)	At5g48880	3-keto-acyl-CoA thiolase 2 (gb AAC17877.1) ; supported by cDNA: gi_3192892_gb_AF062590.1_AF062590	800.679	449.614	0.56154089	378.395	211.409	0.55869924	334.975	194.646	0.5810762
low-temperature-responsive protein 78 (LTI78) / desiccation-responsive protein 29A (RD29A)	At5g52310	low-temperature-induced protein 78 (sp Q06738) ; supported by cDNA: gi_348691_gb_L22567.1_ATHCOR78A	2863.279	863.511	0.30158116	7374.733	928.208	0.12586327	8723.203	4291.044	0.49191151
zinc finger (GATA type) family protein	At5g56860	putative protein similar to unknown protein (pir T04270);supported by full-length cDNA: Ceres:110454.	752.06	414.663	0.55136957	738.796	245.008	0.33163147	355.913	171.773	0.48262637
invertase/pectin methylesterase inhibitor family protein	At5g62360	DC1.2 homologue - like protein DC1.2 homologue, Nicotiana tabacum, EMBL:AB009888	2822.477	459.028	0.16263303	7175.524	600.618	0.08370371	10473.894	3146.858	0.30044776
pectate lyase family protein	At5g63180	pectate lyase ; supported by cDNA: gi_16648839_gb_AY058197.1_	873.48	578.486	0.66227733	438.135	281.278	0.64198934	599.754	361.076	0.60204017
expressed protein	At5g65300	unknown protein ; supported by cDNA: gi_13877834_gb_AF370180.1_AF370180	759.923	319.245	0.42010177	101.545	37.761	0.37186469	348.725	90.587	0.25976629

Appendices

81 cold-inducible non-misregulated genes

These genes showed at least a 1.5-fold induction in wild type plants compared to ambient in microarray experiments (Affymetrix gene chip GEO reference:GSE6167).

Wild type (WT) and *sfr6-1* gene expression data is from the Affymetrix gene chip GEO reference GSE6167. The *sfr6-2* and *sfr6-3* data is from the Affymetrix gene chip GEO reference GSE46084. A gene was said to be non-misregulated in *sfr6* if the ratio of all three *sfr6* alleles (*sfr6-1*, *sfr6-2* and *sfr6-3*) to wild type was less than 0.7 in all 3 *sfr6* alleles compared to wild type.

Gene	Accession	Description	WT1 cold	<i>sfr6-1</i> cold	<i>sfr6-1</i> /WT1 ratio	WT2 cold	<i>sfr6-2</i> cold	<i>sfr6-2</i> /WT2 ratio	WT3 cold	<i>sfr6-3</i> cold	<i>sfr6-3</i> /WT3 ratio
GCN5-related N-acetyltransferase (GNAT) family protein	At1g03150	unknown protein Belongs to PF 00583 Acetyltransferase (GNAT) family; supported by full-length cDNA: Ceres: 10117.	292.364	256.954	0.8788	225.514	252.519	1.119	222.403	237.105	1.0661
expressed protein	At1g03260	hypothetical protein predicted by genscan	142.572	143.958	1.00972	91.793	102.518	1.116838	125.916	118.454	0.940738
acid phosphatase class B family protein	At1g04040	unknown protein Similar to acid phosphatase; Location of ESTs 110C2T7 , gb T42036, and 110C2XP, gb AI100245; supported by cDNA: gi_13926197_gb_AF370572.1_AF370572	2416.849	2382.966	0.9859805	680.144	790.602	1.162403	1543.454	1521.147	0.9855
eukaryotic translation initiation factor 2 subunit 3, putative / eIF2S3, putative / eIF-2-gamma, putative	At1g04170	putative translation initiation factor eIF-2, gamma subunit similar to gb U37354 from S. pombe. ESTs gb T41979, gb N37284 and gb N37529 come from this gene;supported by full-length cDNA: Ceres:37699.	1655.607	1731.803	1.046022	1188.36	1304.854	1.098029	1288.875	1397.469	1.0842
dehydration-responsive protein-related	At1g04430	ankyrin-like protein EST gb ATTS0956 comes from this gene;supported by full-length cDNA: Ceres:108617.	1818.533	1647.475	0.90593	2392.518	2148.778	0.8981	1666.415	1592.615	0.9557
phosphoribosylamine--glycine ligase (PUR2)	At1g09830	putative phosphoribosylglycinamide synthetase Identical to A. thaliana PUR2 (gb X74766). ESTs gb ATTS3927,gb N96446 come from this gene; supported by cDNA:	385.927	403.544	1.04564	342.284	323.699	0.9457	422.751	410.618	0.9712

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		gi_15292772_gb_AY050820.1_									
S-locus lectin protein kinase family protein	At1g11330	receptor-like protein kinase, putative similar to receptor-like protein kinase GB:AAC95353 GI:4008010 from [Arabidopsis thaliana]	117.35	104	0.8862	53.149	54.995	1.0347	40.532	41.27	1.0182
eukaryotic translation initiation factor, putative (EIF4B5)	At1g13020	unknown protein EST gb T22808 comes from this gene	405.092	410.236	1.0126	395.486	423.199	1.0700	507.905	579.464	1.14089
EF-1-alpha-related GTP-binding protein, putative	At1g18070	guanine nucleotide regulatory protein, putative similar to guanine nucleotide regulatory protein GI:3461880 from [Mus musculus]	1288.624	1213.348	0.94158	864.917	787.43	0.9104	953.377	838.93	0.8799562
sulfate adenylyltransferase 2 / ATP-sulfurylase 2 (ASA1) (MET3-1) (APS2)	At1g19920	sulfate adenylyltransferase identical to sulfate adenylyltransferase GI:487404 from [Arabidopsis thaliana];supported by full-length cDNA: Ceres:21320.	1024.685	1052.922	1.02755	833.911	817.986	0.9809	894.651	991.55	1.1083
tRNA pseudouridine synthase family protein	At1g20370	hypothetical protein contains Pfam profile: PF01416 tRNA pseudouridine synthase; supported by cDNA: gi_14334543_gb_AY035176.1_	249.882	242.085	0.96879	87.432	97.078	1.1103	111.892	124.492	1.1126
expressed protein	At1g21050	hypothetical protein predicted by genemark.hmm;supported by full-length cDNA: Ceres:3200.	1269.932	1444.862	1.13774	746.49	821.538	1.1005345	797.744	731.956	0.9175
phosphoribosylanthranilate isomerase 1 (PAI1)	At1g29410	phosphoribosylanthranilate isomerase identical to GI:619749 from [Arabidopsis thaliana] (Plant Cell 7 (4), 447-461 (1995))	435.113	372.346	0.85574	215.479	221.865	1.0296363	167.577	193.639	1.1555
GDSL-motif lipase/hydrolase family protein	At1g29660	lipase/hydrolase, putative contains Pfam profile: PF00657 Lipase/Acylhydrolase with GDSL-like motif;supported by full-length cDNA: Ceres:6680.	3950.271	4412.087	1.11690	2547.214	2631.936	1.0332	3720.111	3638.401	0.9780356
armadillo/beta-catenin repeat family protein	At1g51350	hypothetical protein predicted by genscan	282.348	263.12	0.93189	231.125	222.689	0.9635	267.792	240.639	0.8986
Ras-related GTP-binding protein, putative	At1g52280	GTP-binding protein RAB7D, putative similar to GI:1370187 from [Lotus japonicus] (Plant J. 11 (2), 237-250 (1997)); supported by cDNA: gi_15718409_dbj_AB071847.1_AB071847	679.842	606.51	0.8921	659.293	695.798	1.0553	750.089	808.834	1.0783
mitochondrial import inner membrane	At1g61570	unknown protein similar to small zinc finger-like protein GI:5107149 from [Oryza sativa];	1347.003	1372.053	1.01859	637.62	738.378	1.1580	715.825	708.1	0.9892

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translocase (TIM13)		supported by full-length cDNA: Ceres: 21075.									
DNA-binding storekeeper protein-related	At1g61730	unknown protein similar to hypothetical protein GI:7267126 from [Arabidopsis thaliana];supported by full-length cDNA: Ceres:38650.	1858.849	1845.793	0.99297	1023.284	887.887	0.8676	1317.656	1396.122	1.0595
ABC transporter family protein	At1g64550	ABC transporter protein, putative similar to ABC transporter protein GB:AAF31030 GI:6899653 from [Leishmania major]	649.038	643.748	0.99184	579.852	573.349	0.9887	552.971	506.769	0.9164477
auxin transport protein, putative (PIN3)	At1g70940	auxin transport protein REH1, putative similar to auxin transport protein REH1 GI:3377509 from [Oryza sativa]; supported by cDNA: gi_5817300_gb_AF087818.1_AF087818	1161.138	1120.688	0.96516	1141.021	1105.711	0.96905	494.987	432.207	0.8731
asparaginyl-tRNA synthetase, cytoplasmic, putative	At1g70980	asparaginyl-tRNA synthetase(SYNC1) protein,putative similar to SYNC1 protein GI:5670315 from [Arabidopsis thaliana]	779.871	769.134	0.98623	1189.228	1358.666	1.1424773	1175.019	1232.573	1.0489
galactosyl transferase GMA12/MNN10 family protein	At1g74380	putative alpha galactosyltransferase similar to alpha galactosyltransferase GB:CAB52246 [Trigonella foenum-graecum] (plant cell wall matrix polysaccharide biosynthesis)	713.138	662.793	0.92940	480.794	475.186	0.9883	528.891	481.551	0.9104
zinc knuckle (CCHC-type) family protein	At1g75560	DNA-binding protein similar to cellular nucleic acid binding protein GB:CAA45345 GI:50471 from [Mus musculus]; supported by full-length cDNA: Ceres: 125348.	650.371	657.483	1.01093	480.092	526.507	1.0966	402.099	434.274	1.0800
NAD-dependent epimerase/dehydratase family protein	At1g78570	dTDP-glucose 4,6-dehydratase, putative similar to dTDP-glucose 4,6-dehydratase GI:5921157 from [Streptomyces avermitilis]; supported by cDNA: gi_14596090_gb_AY042833.1_	1526.002	1519.165	0.99551	2046.142	1797.117	0.8782	2067.215	1997.878	0.9664
prefoldin-related KE2 family protein	At2g07340	hypothetical protein predicted by genefinder	211.121	196.44	0.93046	153.162	154.013	1.0055562	191.076	183.763	0.9617272
small nuclear ribonucleoprotein E, putative / snRNP-E, putative	At2g18740	putative small nuclear ribonucleoprotein E ;supported by full-length cDNA: Ceres:24619.	657.246	626.497	0.9532153	422.662	371.042	0.8778693	489.01	417.155	0.8530602
violaxanthin de-epoxidase-related	At2g21860	unknown protein predicted by gencan;supported by full-length cDNA: Ceres:38497.	1052.848	1059.407	1.0062297	555.568	626.439	1.1275649	714.127	686.649	0.9615222

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trehalose-6-phosphate phosphatase, putative	At2g22190	putative trehalose-6-phosphate phosphatase	773.974	666.881	0.8616323	144.043	152.599	1.0593989	633.976	580.024	0.9148989
aminotransferase class I and II family protein	At2g22250	putative aspartate aminotransferase ;supported by full-length cDNA: Ceres:112880.	666.395	672.536	1.0092152	484.769	557.055	1.1491143	310.179	333.394	1.0748438
homeobox-leucine zipper protein 6 (HB-6) / HD-ZIP transcription factor 6	At2g22430	homeodomain transcription factor (ATHB-6) ; supported by cDNA: gi_16974586_gb_AY060569.1_	1810.476	1821.222	1.0059354	953.698	961.603	1.0082887	1670.315	1808.323	1.0826239
splicing factor, putative	At2g24590	putative RSZp22 splicing factor	926.659	954.457	1.0299980	788.943	805.081	1.0204552	739.028	670.917	0.907837
mitochondrial import inner membrane translocase (TIM10)	At2g29530	unknown protein ; supported by cDNA: gi_5107173_gb_AF150093.1_AF150093	883.214	975.894	1.1049331	458.865	477.252	1.0400706	652.348	623.786	0.9562166
aspartate aminotransferase, mitochondrial / transaminase A (ASP1)	At2g30970	aspartate aminotransferase (AAT1) identical to GB:U15026;supported by full-length cDNA: Ceres:34360.	1358.587	1473.269	1.0844127	771.441	685.343	0.8883932	635.767	672.734	1.0581455
delta 9 desaturase (ADS2)	At2g31360	delta 9 desaturase ALMOST identical (4 aa diff't) to GP:2970036;supported by full-length cDNA: Ceres:21841.	4481.876	4108.329	0.9166538	5965.467	5108.593	0.8563609	6677.861	6629.054	0.9926912
40S ribosomal protein S12 (RPS12C)	At2g32060	40S ribosomal protein S12 ;supported by full-length cDNA: Ceres:13453.	3322.54	3396.288	1.0221962	2419.995	2692.206	1.1124841	3065.28	2683.331	0.8753950
serine carboxypeptidase S10 family protein	At2g35770	putative serine carboxypeptidase II	129.51	132.888	1.0260829	45.249	51.71	1.1427876	200.912	194.997	0.9705592
phenylalanine ammonia-lyase 1 (PAL1)	At2g37040	phenylalanine ammonia lyase (PAL1) ; supported by cDNA: gi_15028192_gb_AY045919.1_	2552.947	2614.556	1.0241325	3622.19	3205.438	0.8849447	3741.437	3461.518	0.9251840
adenylate kinase family protein	At2g37250	putative adenylate kinase ;supported by full-length cDNA: Ceres:15831.	691.515	685.506	0.9913103	691.885	666.839	0.9638003	806.501	715.758	0.8874855
zinc finger (CCCH-type) family protein	At2g40140	putative CCCH-type zinc finger protein also an ankyrin-repeat protein	816.746	849.541	1.0401532	613.589	549.651	0.8957967	674.511	711.86	1.0553719
AP2 domain-containing transcription factor, putative (DRE2B)	At2g40350	AP2 domain transcription factor	324.978	331.739	1.0208044	63.284	69.296	1.0950002	95.274	94.39	0.9907215
chaperonin, putative	At3g02530	putative chaperonin similar to chaperonin subunit 6a (zeta) GB:NP_033968 from [Mus musculus];supported by full-length cDNA:	1028.997	1024.635	0.9957609	801.784	923.74	1.1521058	920.312	882.673	0.9591011

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		Ceres:116386.									
guanylate kinase, putative	At3g06200	putative guanylate kinase similar to guanylate kinase (GmK) GB:AAD31506 [Salmonella typhimurium]; contains Pfam profile: PF00625 guanylate kinase; supported by cDNA: gi_14190398_gb_AF378877.1_AF378877	181.276	196.321	1.0829949	273.223	278.664	1.0199141	326.577	376.887	1.1540524
60S ribosomal protein L29 (RPL29B)	At3g06680	ribosomal protein L29, putative similar to 60S ribosomal protein L29 GB:P25886 from [Rattus norvegicus]	2163.351	2227.498	1.0296516	1399.234	1627.458	1.1631063	1748.415	1697.022	0.9706059
60S ribosomal protein L29 (RPL29A)	At3g06700	ribosomal protein L29, putative similar to ribosomal protein L29 GI:7959366 (Panax ginseng);supported by full-length cDNA: Ceres:315.	3176.562	3180.62	1.0012774	2221.594	1924.022	0.8660547	2399.864	2135.584	0.8898770
sterile alpha motif (SAM) domain-containing protein	At3g07170	unknown protein ; supported by cDNA: gi_15294217_gb_AF410300.1_AF410300	447.998	416.621	0.9299617	510.535	436.476	0.8549384	522.446	500.409	0.9578196
60S acidic ribosomal protein P0 (RPP0C)	At3g11250	60S acidic ribosomal protein, putative similar to 60S acidic ribosomal protein P0 GI:2088654 [Arabidopsis thaliana];supported by full-length cDNA: Ceres:38036.	1506.267	1431.657	0.9504669	840.242	752.404	0.8954610	824.606	897.693	1.0886326
DNA-binding protein, putative	At3g11580	putative DNA binding protein similarity to RAV2 DNA binding protein GB:BAA34251 [Arabidopsis thaliana]	113.917	116.531	1.0229465	77.198	80.747	1.0459729	101.241	91.183	0.9006529
expressed protein	At3g18790	unknown protein	556.756	544.064	0.9772036	380.531	414.387	1.0889704	338.349	389.535	1.1512816
sulfotransferase family protein	At3g45070	sulfotransferase-like protein FLAVONOL 4 - SULFOTRANSFERASE - Flaveria chloraefolia, EMBL:M84136	117.763	100.266	0.8514219	21.785	22.511	1.0333256	32.73	35.777	1.0930950
Ras-related protein (RAB11A) / small GTP-binding protein, putative	At3g46830	GTP-binding protein Rab11 ; supported by full-length cDNA: Ceres: 35596.	441.963	458.09	1.036489	385.379	386.391	1.0026259	423.727	485.243	1.1451783
expressed protein	At3g52740	hypothetical protein ; supported by cDNA: gi_15450654_gb_AY052695.1_	1283.889	1183.907	0.9221256	425.227	432.637	1.0174259	564.227	555.178	0.9839621
phosphoribosylformylglycinamidine cyclo-ligase, chloroplast / phosphoribosyl-	At3g55010	phosphoribosylformylglycinamidine cyclo-ligase precursor ; supported by cDNA: gi_16974614_gb_AY060585.1_	858.519	783.364	0.9124597	458.328	492.38	1.0742961	475.526	474.299	0.9974197

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aminoimidazole synthetase / AIR synthase (PUR5)											
sugar transporter family protein	At4g00370	hypothetical protein	1474.787	1361.844	0.9234174	1465.477	1578.642	1.0772205	1084.355	1057.835	0.9755430
glycosyltransferase family protein	At4g09500	putative protein anthocyanin rhamnosyltransferase -Petunia x hybrida, PID:g454253	304.382	290.77	0.9552798	69.514	66.758	0.9603533	146.155	137.31	0.9394820
expressed protein	At4g14240	hypothetical protein	480.542	484.977	1.0092291	264.666	238.621	0.9015929	239.74	267.831	1.1171727
eukaryotic translation initiation factor 4E 1 / eIF-4E1 / mRNA cap-binding protein 1 (EIF4E1)	At4g18040	translation initiation factor eIF4E ;supported by full-length cDNA: Ceres:25447.	1768.628	1707.986	0.9657124	768.029	820.308	1.0680690	842.444	952.815	1.1310128
adenine phosphoribosyltransferase, putative	At4g22570	adenine phosphoribosyltransferase (EC 2.4.2.7) - like protein adenine phosphoribosyltransferase, Triticum aestivum, T06263; supported by full-length cDNA: Ceres: 11009.	1189.523	1309.578	1.100927	1346.758	1205.626	0.8952061	769.012	690.443	0.8978312
expressed protein	At4g25210	putative protein cyclin II - human, PID:g758587; supported by cDNA: gi_14423517_gb_AF386996.1_AF386996	945.114	969.576	1.0258825	748.573	776.975	1.0379415	809.815	885.415	1.0933546
amine oxidase family protein	At4g29720	putative protein Cs protein, Drosophila melanogaster, AF091328;supported by full-length cDNA: Ceres:158028.	1634.15	1899.24	1.1622188	1101.131	992.832	0.9016474	1309.693	1144.764	0.8740704
expressed protein	At4g32020	putative protein	2633.813	2970.008	1.1276457	3377.027	2968.707	0.8790889	3546.801	3064.533	0.8640273
beta-galactosidase, putative / lactase, putative	At4g36360	beta-galactosidase like protein ; supported by cDNA: gi_15810492_gb_AY056285.1_	1328.959	1247.171	0.9384570	841.924	939.107	1.1154296	1223.021	1253.381	1.0248237
DNAJ heat shock N-terminal domain-containing protein	At4g39150	dnaJ-like protein CAJ1 protein, Saccharomyces cerevisiae, PIR2:S48085	712.752	751.062	1.0537494	635.898	553.912	0.8710705	574.79	641.662	1.1163416
glycine-rich RNA-binding protein 8 (GRP8) (CCR1)	At4g39260	glycine-rich protein (clone AtGRP8) ; supported by cDNA: gi_166838_gb_L00649.1_ATHRBPB	3236.631	2969.954	0.917606	2590.706	2970.159	1.1464670	2853.955	3077.036	1.0781655
stress-responsive protein, putative	At5g01410	pyridoxine biosynthesis protein - like pyridoxine biosynthesis protein pyroA - Emericella nidulans;supported by full-length	3543.37	3984.101	1.1243818	5242.777	4773.358	0.9104636	4309.031	4331.316	1.0051717

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		cDNA: Ceres:8877.									
40S ribosomal protein S15 (RPS15D)	At5g09510	ribosomal protein S15-like ribosomal protein S15 - Arabidopsis thaliana, EMBL:Z23161;supported by full-length cDNA: Ceres:164.	1487.457	1525.519	1.0255886	846.97	910.44	1.0749377	1125.132	963.812	0.8566217
eukaryotic translation initiation factor SUI1 family protein	At5g11900	putative protein density regulated protein drp1, Homo sapiens, EMBL:AF038554	396.115	368.091	0.9292528	258.894	292.747	1.1307600	271.342	275.285	1.0145314
MA3 domain-containing protein	At5g17930	unknown protein	196.643	168.958	0.8592118	121.326	135.695	1.1184329	152.517	175.434	1.1502586
ribosomal protein L36 family protein	At5g20180	ribosomal protein L36-like ribosomal protein L36 - Synechocystis sp., PIR:S77481;supported by full-length cDNA: Ceres:28109.	967.667	1015.795	1.0497361	633.863	571.984	0.9023779	874.63	871.325	0.9962212
expressed protein	At5g28040	putative protein various predicted proteins, Arabidopsis thaliana and Oryza sativa	563.307	488.387	0.8669997	260.144	225.271	0.8659473	273.357	264.743	0.9684880
expressed protein	At5g28430	putative protein predicted proteins - Arabidopsis thaliana	77.112	73.534	0.9535959	81.951	91.82	1.1204256	84.326	87.166	1.0336788
expressed protein	At5g41810	unknown protein ;supported by full-length cDNA: Ceres:126660.	723.797	696.125	0.9617682	185.987	206.297	1.1092011	299.908	314.262	1.0478613
expressed protein	At5g42060	putative protein similar to unknown protein (gb AAF19669.1)	303.132	289.697	0.9556793	90.759	91.519	1.0083738	115.301	109.054	0.9458200
nuclear RNA-binding protein, putative	At5g47210	putative protein similar to unknown protein (pir G71444);supported by full-length cDNA: Ceres:19104.	3136.34	2924.282	0.9323867	1720.33	1854.818	1.0781757	1602.125	1555.726	0.9710390
heat shock protein 70 / HSP70 (HSC70-7)	At5g49910	heat shock protein 70 (gb AAF27639.1) ; supported by cDNA: gi_6746591_gb_AF217459.1_AF217459	2247.32	2444.277	1.0876408	2090.948	1970.203	0.9422537	1333.875	1320.419	0.9899121
mitochondrial import inner membrane translocase (TIM8)	At5g50810	small zinc finger-like protein ;supported by full-length cDNA: Ceres:33833.	1023.742	981.278	0.9585207	642.765	659.184	1.0255443	719.063	740.629	1.0299911
fibrillarin 1 (FBR1) (FIB1) (SKIP7)	At5g52470	fibrillarin 1 (AtFib1) identical to fibrillarin 1 GI:9965653 from [Arabidopsis thaliana]; C-terminus identical to SKP1 interacting partner 7 GI:10716959 from [Arabidopsis thaliana]; supported by cDNA: gi_10716958_gb_AF263383.1_AF263383	2743.645	3084.725	1.1243163	3330.269	3830.273	1.1501392	3224.583	3376.433	1.0470913
RabGAP/TBC domain-	At5g53570	GTPase activator protein of Rab-like small	313.99	300.93	0.9584063	348.532	348.647	1.0003299	392.49	369.511	0.9414532

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containing protein		GTPases-like protein ; supported by cDNA: gi_14517421_gb_AY039546.1_									
auxin-responsive family protein	At5g53590	unknown protein	509.562	501.947	0.9850557	179.977	170.274	0.9460875	335.592	376.871	1.1230035
heat shock protein 81-2 (HSP81-2)	At5g56030	HEAT SHOCK PROTEIN 81-2 (HSP81-2) (sp P55737)	2526.584	2846.955	1.1268000	3555.103	4059.669	1.1419272	2327.221	2401.02	1.0317112
CBL-interacting protein kinase 10 (CIPK10)	At5g58380	serine/threonine protein kinase ; supported by cDNA: gi_13249118_gb_AF295665.1_AF295665	441.336	504.536	1.1432015	243.686	272.863	1.1197319	289.767	290.728	1.0033164
DNA-directed RNA polymerase II	At5g59180	RNA polymerase II	531.113	492.533	0.9273600	274.513	287.042	1.0456408	413.72	370.772	0.8961906

Appendix III: Primer Regression Coefficients

Regression Coefficient of primer viability for genes used in qRT-PCR experiments. Primer sequences can be found in Materials and Methods section **II.9.2. Primers used for transcript analysis.**

